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EDITORS' NOTE

In this issue we publish the lectures from two recipients of the Silverstein-Simeone Lecture Award. Ian Baldwin received the 1998 award, and Louise Vet received the award in 1996. The lectures were presented at the annual meeting of the International Society of Chemical Ecology in the year the award was made. We will publish other lectures in coming issues.

We call attention to changes on the inside front and back cover relative to Editorial Board composition and Instructions to Contributors, respectively. Since the founding of the journal in 1974 by John Simeone and Milt Silverstein, chemical ecology has matured into a strong discipline practiced by many scientists worldwide, and the board needs more balance internationally. We believe also that it is time to recognize more of the talent available by initiating rotating board membership. We invite new board members to serve for a five-year term, and ask those continuing members to stay three more years for continuity. The hardest part of the process was asking members to retire from the board. This we did not do lightly; we acknowledge the important contribution of all the board members, and we are grateful for their help over the years.

On the inside back cover we make new requests of authors that we anticipate will facilitate the processing of papers and help us publish faster.

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INDUCIBLE NICOTINE PRODUCTION IN NATIVE *Nicotiana* AS AN EXAMPLE OF ADAPTIVE PHENOTYPIC PLASTICITY[1]

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Abstract—Nicotine, an inducible defense in a number of *Nicotiana* species, exemplifies adaptive phenotypic plasticity. The mechanisms responsible for its production are reviewed, and the induced character states are characterized allometrically in order to understand how inducibility changes over ontogeny responds to environmental variables that influence plant growth, and to relate inducible production to plant fitness correlates. The empirical evidence for fitness costs and benefits of inducible nicotine production are considered, and the physiological and ecological mechanisms potentially responsible for the costs are considered. An intimate understanding of the plant's natural history is an essential prerequisite to understanding these costs and benefits. Inducible nicotine production is just one of many traits that are altered after herbivore attack, and the cost–benefit model provides a valuable heuristic framework in which to understand the selective factors responsible for the maintenance of inducibly expressed traits.

Key Words—Phenotypic plasticity, induced defense, nicotine, costs and benefits, post-fire germination.

INTRODUCTION

The environment plays two essential roles in an organism's life: (1) as a selective agent culling the phenotypes not well matched to a particular environment, and (2) as a manipulator of phenotypic expression from a given genotype (Scheiner, 1993). This dual role, as provider of the fitness function (B, Figure 1) and as the force that fits the phenotype to the environment (A, Figure 1),

[1]ISCE Silverstein–Simeone Lecture Award, 1998.

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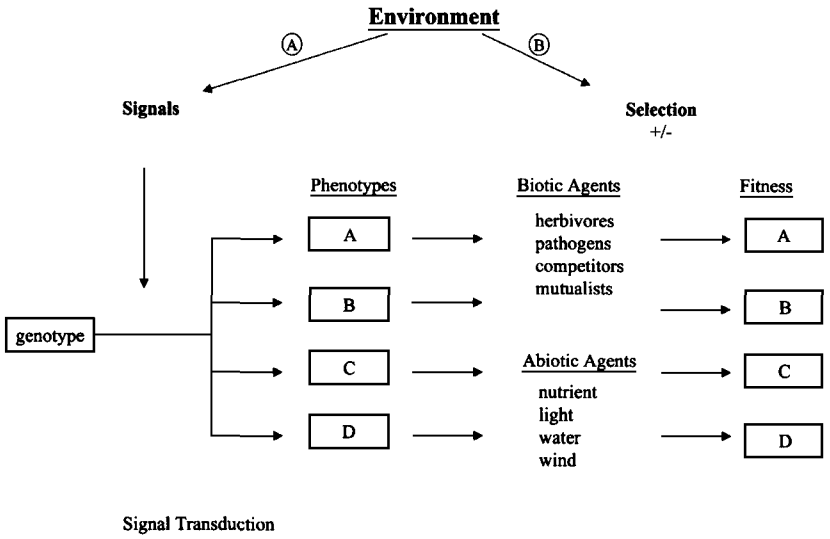

FIG. 1. The environment plays two roles in the life of every organism: (A) as a provider of signals that organisms use to modify their phenotypes via various signal transduction cascades and (B) as a provider of the fitness functions (such as biotic and abiotic agents of natural selection) that select those phenotypes most fit in a particular environment (adapted from Scheiner, 1993b). Phenotypic plasticity is a major evolutionary response of sedentary organisms to environmental variation.

highlights the tension between the study of phenotypic plasticity and the neo-Darwinian synthesis, which has eschewed environmental effects on variation, perhaps to avoid Lamarckian associations (Sultan, 1992). While phenotypic plasticity is a major evolutionary response of sedentary and modular organisms to environmental variability (Schlichting, 1986; Thompson, 1991; Scheiner, 1993), if a given genotype could produce a number of truly protean phenotypes, responding adaptively to all environmental variation, then the phrase "survival of the fittest" would be a meaningless construct. However, organisms are not infinitely plastic and their phenotypic responses to environmental variation are frequently not adaptive; thus it is of interest to understand the factors which constrain plasticity.

The costs of plasticity and the limits to its benefits have been reviewed recently (DeWitt et al., 1998), and the costs most frequently considered are the time lags between the environmental signal and the expression of the phenotype, the maintenance of the regulatory and sensory machinery required for plasticity, and the lack of reliability of the information that organisms use to modulate their phenotypes. Particularly in regard to the third consideration, the discipline

of chemical ecology has contributed substantially to the understanding of the evolution of phenotypic plasticity.

The characterization, synthesis, and quantification of the chemical signals that organisms utilize in their interactions with their environment provide researchers with valuable insights into the information content of these signals. Perhaps equally important are the experimental manipulations that these signals enable. They provide researchers with tools to uncouple a response from the ecologic interaction that normally elicits it, allowing them to rigorously test if the responses are adaptive (i.e., whether the elicited phenotype "fits" its environment better than other realistic alternative phenotypes). Moreover, a knowledge of the signal transduction pathways that organisms use to alter their phenotypes in response to environmental signals suggests additional tools that can be used to experimentally produce sets of realistic alternative phenotypes (Figure 1). The creation of "phenotypic sets" within which the adaptive significance of phenotypic variation can be rigorously tested is a technique frequently used by behaviorists (Reeve and Sherman, 1993) but not commonly by researchers studying plastic responses (see Schmitt et al., 1995, for an excellent example). An understanding of how these signal cascades transduce the perception of environmental signals into phenotypic changes can also provide important insights into how an organism filters the information it receives from its environment.

Herbivores have altered the expression of many perceived plant defense traits over evolutionary time and can alter the expression of many of these same traits in ecological time (Karban and Myers, 1989; Tallamy and Raupp, 1991; Karban and Baldwin, 1997). Many plant traits thought to function as direct or indirect defenses against herbivore attack, or to allow plants to tolerate fitness consequences of herbivore attack, are altered by herbivore attack, and many of these changes are mediated by components of a common signal transduction cascade (Baldwin, 1999). For example, traits such as the production of toxins, which may function directly as defenses, or the release of volatiles, which may function as indirect defenses (Turlings et al., 1995; Takabayashi and Dicke, 1996), are induced after herbivore attack in part due to the activation of the jasmonate (also called the octadecanoid, or Vick-Zimmerman) cascade (Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Baldwin, 1999). This cascade produces a family of active metabolites (Blechert et al., 1995) with similarities in structure, function, and biosynthetic origins to the prostaglandins, prostacyclins, and leukotrienes of animals and may function as a "master switch," reconfiguring plant phenotypes in response to environmental signals (Bergey et al., 1996). Hence, there is substantial interest in determining whether the traits that are activated by the jasmonate cascade are examples of adaptive phenotypic plasticity.

A "phytocentric" analysis of induced responses—one that characterizes the consequences of the induced responses for plant fitness, as well as the traits thought to be responsible for the fitness effects—is essential if these traits are to

be understood as examples of phenotypic plasticity. Since plant fitness correlates, namely the production of viable seed and the siring of offspring, are whole-plant traits reflecting the culmination of vegetative growth and reproductive maturation of the phenotype, these "developmental reaction norms" of a defensive trait are best established with an allometric analysis (Pigliucci et al., 1996; Baldwin, 1997).

Interestingly, while the phenomenon of induced resistance has been demonstrated in more than 110 plant–herbivore associations (Karban and Baldwin, 1997), most of these examined the response from the herbivore's perspective (e.g., effects on the herbivore's growth and population dynamics). Only two recent studies have demonstrated a fitness benefit to the plant of an induced response in nature (Agrawal, 1998; Baldwin, 1998), and thus only these two can claim that an induced response exemplifies adaptive phenotypic plasticity. However, in one of these studies [in which *Raphanus sativus* plants, exposed to early-season herbivory by larvae of *Pieris rapae*, received less subsequent damage and produced heavier seed than did mechanically damaged controls (Agrawal, 1998)], it is not clear that a plant trait caused the apparent induced resistance. Disease or factors directly associated with the early-season herbivory, rather than a plant response (Fowler and Lawton, 1985), could have produced the fitness effect. In summary, the induced traits should be characterized from the perspective of the organism producing the trait.

A detailed understanding of the natural history of the organism exhibiting conditional behaviors is essential to generating realistic, testable hypotheses about the fitness costs and benefits maintaining the responses in natural populations. Inducible defenses are inherently inferior to defenses that are constitutively deployed because of the time-lag between the first attack and the activation of the defense. As a result of this delay, a plant remains vulnerable for hours or even days while waiting for the defense to be activated. The cost–benefit model argues that although defenses are beneficial and may increase a plant's fitness when it is under attack, they are costly when they are not needed, which decreases plant fitness. In short, inducibility is thought to be a cost-savings measure, allowing plants to time the production of a defense with the prevailing environmental conditions and to forgo the payment of defense costs when they are not needed.

While the demonstration of "costs" in a fitness currency is relatively straightforward—documenting fitness reductions associated with producing the "wrong" phenotype in the wrong environment—and only requires techniques for eliciting the phenotype in a physiologically realistic fashion, understanding the mechanisms responsible for these fitness reductions is not so simple. The processes responsible for a costly trait can occur at many different levels, from the physiological (resource or autotoxicity costs) to the ecological. The most commonly invoked mechanisms responsible for these trade-offs are resource-based trade-offs that would occur if the plant's production of chemicals (or other

traits, such as trichomes) for defense utilizes a fitness-limiting resource (carbon, nitrogen, etc.) that otherwise could be used for growth and reproduction. However, the requirements for establishing a resource-based trade-off are onerous and absent from all studies that have tried to examine this topic (Mole, 1994). While many studies have documented negative relationships between plant growth and defense (reviewed in Herms and Mattson, 1992), none has demonstrated that a resource-based trade-off between the different plant functions was responsible for the negative growth-defense relationship. Perhaps as a response to the difficulties of empirically examining resource-based trade-offs, ecological researchers are turning their attention to other types, such as "ecological" trade-offs between resistance and susceptibility to different natural enemies (Simms, 1992; Adler and Karban, 1994; Rausher, 1996), mutualists (Euler and Baldwin, 1996; Strauss, 1997), or between resistance and tolerance to herbivory (van der Meijden et al., 1988; Rosenthal and Kotanen, 1994; Fineblum and Rausher, 1995; Mauricio et al., 1997). The plethora of costs that could be responsible for maintaining inducible resistance makes the cost-benefit model both broadly applicable but also difficult to falsify (Simms, 1992; Karban and Baldwin, 1997). Moreover, alternative models to the cost–benefit model exist [e.g., the moving target (Karban, 1993; Adler and Karban, 1994; Karban and Baldwin, 1997)], which argue that the ability to produce a variable phenotype is in itself a defensive trait and do not propose that the well-defended phenotype is costly; hence, if we are to understand the evolution and maintenance of phenotypic plasticity, it is essential to determine whether fitness costs are relevant.

The production of the toxic alkaloid nicotine in two native species of *Nicotiana* (*N. sylvestris* and *N. attenuata*) illustrates adaptive phenotypic plasticity. The overarching goal of my research effort has been to understand the mechanisms responsible for nicotine induction in sufficient detail to allow for manipulations of the responses under field conditions and thereby test its fitness consequences. Most of the physiological work has been performed with *N. sylvestris*, but political instability in its home range made it difficult to conduct fieldwork, so the research effort switched to the North American species, *N. attenuata*, to which the major mechanistic discoveries from *N. sylvestris* also apply. The nornicotine- (*N. repanda* and *N. trigonophylla*) and anabasine-accumulating (*N. glauca*) species found in North American also exhibit dramatic wound-induced increases in their dominant alkaloids (Baldwin and Ohnmeiss, 1993), and many of the patterns may apply to these *Nicotiana* species as well. This review takes a "phytocentric" view of induced nicotine production by examining inducibility at a number of different temporal and spatial scales to better understand the trait from the plant's perspective. Additionally, it considers the fitness consequences of inducible nicotine production and its responsible mechanisms and how these mechanisms may have influenced other life history traits.

PHYSIOLOGICAL CHARACTERIZATION

Within-Plant Distribution, Production, and Metabolism. Both *Nicotiana* species are annuals and accumulate nicotine at 0.1–1.0% leaf dry mass in undamaged plants. After real or simulated folivory to plants grown in the glasshouse (Baldwin, 1988a–c, 1989) or in natural populations (Baldwin and Ohnmeiss, 1993; Baldwin, 1998), nicotine concentrations increase dramatically—typically four fold or, under some circumstances, 10-fold—to attain concentrations that are sufficient to deliver what is for many herbivores a lethal dose in a single meal and also protect induced tissues from herbivores that are adapted to nicotine (Baldwin, 1988c, 1991). Optimal defense theory (McKey, 1979; Zangerl and Bazzaz, 1992) argues that defense metabolites are distributed within plants so that their fitness benefits are optimized in light of their fitness costs and are allocated preferentially to tissues with high fitness value to the plant and a high probability of attack. Consistent with these predictions are the observations that young leaves, stems, and reproductive parts tend to have the highest concentrations of nicotine, while roots and old leaves have the lowest (T. E. Ohnmeiss and I. T. Baldwin, unpublished results; Baldwin and Karb, 1995; Euler and Baldwin, 1996; Ohnmeiss et al., 1997). Particularly high concentrations can be found in the trichomes of *N. attenuata*, which cover the calyx of flowers and developing seed capsules. Here a single trichome can contain between 1.3–3.6 μg of nicotine. In contrast to the calyx, seeds contain little nicotine. In short, the within-plant distribution of nicotine is consistent with the predictions of optimal defense theory.

The results of many experiments support the model that nicotine is largely synthesized in the roots and transported to the shoots in the xylem stream. For example, when the roots and shoots of nicotine-producing and non-nicotine-producing species are grafted together in different combinations, only the combinations with nicotine-producing roots accumulate this alkaloid in substantial quantities (Dawson, 1942; Baldwin, 1991). Leaf damage dramatically increases the amount of nicotine exported from the roots in the xylem stream (Baldwin, 1989) with a large fraction of this nicotine synthesized *de novo* after wounding, as determined by ^{15}N-pulse-chase techniques (Baldwin et al., 1994a). While the activity of root nitrate reductase (Baldwin et al., 1993) and the total amount of nitrogen reduced in the roots (Baldwin et al., 1994a) appears to be unaffected by wounding, the large increases in nicotine concentrations in the xylem fluid leaving roots occur with a concurrent decrease in the export of amino acids and amides, principally glutamate and glutamine (Baldwin et al., 1993). Induced increases in nicotine accumulation are inhibited in plants whose stems have been steam girdled (Baldwin, 1989) or chilled (G. Y. Lynds and I. T. Baldwin, unpublished results) below the leaf damage, the distribution of nicotine's biosynthetic enzymes corroborates the model that nicotine is synthesized primarily in root tis-

sues. Putrescine *N*-methyltransferase (PMT) is likely the key regulatory enzyme in nicotine biosynthesis (Wagner et al., 1986; Feth et al., 1986), roots have higher PMT activity than shoots, and PMT activity is stimulated by damage to the shoot (Mizusaki et al., 1973; Saunders and Bush, 1979; Wagner et al., 1986). With a probe derived from the PMT gene from *N. tabacum* (Hibi et al., 1994), we have cloned *pmt* from *N. attenuata* and determined that *pmt* mRNA is found only in root tissues and that the amount of mRNA increases after damage to the shoot (S. V. Reddy and I. T. Baldwin, unpublished results). In summary, these plants appear to maintain a constant amount of nitrogen reduction and assimilation in the roots, a large proportion of which (as high as 30%) can be directed into nicotine synthesis after wounding and herbivore attack, and the changes appear to be at least partially transcriptionally regulated.

The sequestration of nicotine biosynthesis in the roots results in a large spatial separation between the site of synthesis (roots) and the site of herbivore attack (leaves) and requires a long-distance signal transduction pathway plus a long-distance transport pathway to return the nicotine to the shoots after synthesis. The damage signal is positively correlated with the amount of damage that a plant receives (Baldwin, 1989; Baldwin and Schmelz, 1994). The fact that it can be blocked by steam girdling the stem of the plant below the site of damage suggests that it is phloem-borne; steam kills the phloem but leaves the xylem intact. The damage signal is relatively show moving, taking more than 1 hr to travel through the damaged leaf and increases *de novo* rates of nicotine synthesis in the roots after 10 hr. These rates suggest that the signal is probably not electrical and are consistent with a chemical signal (Baldwin et al., 1994a; Baldwin and Schmelz, 1994).

The results of many experiments demonstrate that jasmonic acid (JA) is an essential component of the signal transduction cascade. JA pools increase rapidly after leaf damage, reach maximum values 90 min after damage, and decay to levels found in undamaged plants within 24 hr. The JA levels in the roots increase more slowly, attaining maximum values approximately 180 min after damage to the leaves (Baldwin et al., 1994b, 1997b). When the amount of damage is quantitatively varied, strong positive relationships exist among the amount of leaf wounding, the changes in JA concentrations, and whole plant nicotine (Baldwin et al., 1997b; Ohnmeiss et al., 1997). In other words, the more a leaf is wounded, the larger the increase in JA in the leaf 90 min after wounding, and the larger the change in whole plant nicotine five days after wounding. Additions of physiologically relevant quantities of JA to roots as methyl jasmonate (MeJA) or JA increase *de novo* nicotine synthesis from $^{15}NO_3$ in a manner similar to that induced by leaf damage (Baldwin, 1996; Baldwin et al., 1996). Additions of MeJA or JA to the shoots mimic both the timing of changes in pool sizes induced by damage and the magnitude of endogenous JA and nicotine synthesis (Baldwin et al., 1994; 1997b). The wound-induced increase in JA is required for the

induced increase in nicotine; if the increase in JA is inhibited by applying any of a number of different lipoxygenase inhibitors (e.g., salicylates, *n*-propyl gallate, antipyrine) or the plant hormone indole acetic acid, directly to the wounded tissues, the nicotine response is similarly inhibited (Baldwin et al., 1996, 1997b). Applications of these inhibitors to unwounded tissues adjacent to the wound do not influence induced nicotine production. In short, wound-induced JA in the leaves is both necessary and sufficient to activate nicotine metabolism.

It is not yet known if the wound-induced increase in JA in the shoot is directly transported to the roots or if some other indirect signal pathway is involved. Carbon-14-labeled JA that is applied to shoots is rapidly metabolized at the site of application, reflecting rapid detoxification, but sufficient quantities of labeled JA are transported to the roots to account for the changes in root JA pools that are observed after wounding or after application of MeJA to the leaves (Zhang and Baldwin, 1997). Moreover, the kinetics of shoot-to-root transport of labeled JA match exactly the changes induced by wounding. Whether endogenously produced JA is transported through the plant is not clear at this time. In tomato, the octadecapeptide systemin stimulates an increase in JA synthesis that culminates in increased proteinase inhibitor synthesis (Farmer and Ryan, 1992; Koiwa et al., 1997). A similar transduction pathway may also be responsible for wound-induced nicotine production. Our current working model for the long-distance signal transduction cascade is that wounding increases JA pools in shoots, which either directly through transport or indirectly through a signal such as systemin, increases JA pools in roots; these, in turn, stimulate nicotine synthesis in the roots and increase nicotine pools throughout the plant (Figure 2).

Allometric Characterization of Induction. From the above considerations, it is clear that induced nicotine production must be considered a whole-plant trait, since the response requires components distributed throughout the plant. These whole-plant changes in nicotine pools are readily described allometrically by regressing the nicotine pool in the plant against the nicotine-free biomass of the plant acquired from a series of harvests at many stages during development, induced with different elicitors, or grown under different nutrient regimes. The slope of this relationship describes how nicotine accumulation changes as plants increase in biomass. Because neither species appreciably metabolizes its endogenously synthesized nicotine (Baldwin et al., 1994a, 1998; Baldwin and Ohnmeiss, 1994b), the slope of the relationship describes how plants partition resources to nicotine production as they grow.

Wound-induced changes are readily quantified as increases in the slope of the relationship. If a plant is wounded once, the slope of its nicotine allometry is dramatically increased, but the increase is short-lived; 10 days after wounding, the slope resembles that of undamaged plants, and the wound-induced increase in nicotine synthesis is reflected in the larger intercept of the allometric relationship

(Ohnmeiss and Baldwin, 1994). If, however, plants are continually stimulated by wounding or with jasmonates (e.g., Figure 3), then plants will continue to grow along their induced allometric relationship and increase their whole-plant concentrations of nicotine over time. These allometric relationships apply not just to vegetative tissues but also to reproductive parts. For example, the reproductive parts of *N. attenuata* plants that had their leaves wounded during vegetative growth moved from a lower allometry to a higher one in response to the wounding (Baldwin and Karb, 1995). The change in allometric setpoints occurred as new reproductive parts are produced with new allometric setpoints; previously produced reproductive buds did not alter their setpoints after damage.

These allometric setpoints are apparently altered only when a plant alters its rate of *de novo* nicotine synthesis. When cultured in a hydroponic solution containing nicotine at concentrations equivalent to those found in the xylem stream (Baldwin et al., 1993), *N. sylvestris* plants rapidly accumulate large quantities of nicotine (Baldwin and Callahan, 1993). However, unlike the wound- or jasmonate-induced increases in nicotine accumulation that result from *de novo* synthesis, these increases are transitory. The plants rapidly metabolize this exogenously acquired nicotine into nornicotine and myosmine, attaining nicotine pools appropriate for the plant's size. If plants are wounded when they are accumulating nicotine from the hydroponic solution, they will overshoot the pool size normally found in wounded plants but rapidly metabolize the excess quantity of nicotine to attain a pool size appropriate for a damaged plant (Baldwin and Ohnmeiss, 1994b). In summary, *Nicotiana* plants have setpoints for their whole-plant nicotine pools, and these setpoints increase when a plant synthesizes a larger amount of nicotine per unit of growth in response to wounding or wound signals.

These allometrically defined setpoints are useful for comparing nicotine production of plants that differ in size, age, or growth rates and allow one to separate the influence of altered ontogeny on nicotine accumulation. For example, both the amount of available nitrogen and its chemical form vary enormously in *N. attenuata*'s natural habitats. This species mass-germinates from long-lived dormant seed banks after a fire and persists for approximately three years after fires (Baldwin et al., 1994c; Lynds and Baldwin, 1998; Preston and Baldwin, 1999). During this time, the soil nitrogen supply can decrease dramatically as post-fire succession and leaching depletes soil reserves (Lynds and Baldwin, 1998). Since both source and supply of nitrogen will dramatically influence plant growth rates and development, we used an allometric analysis to understand how variations in nitrogen source and supply influence nicotine production. By altering nitrate supply rates eightfold (Figure 3, top) in hydroponic culture, we simulated these alterations in available soil nitrogen. In addition, since the form of nitrogen available to plants also changes during postfire succession as soil ammonia pools are biologically nitrified to nitrate (Lynds and Baldwin,

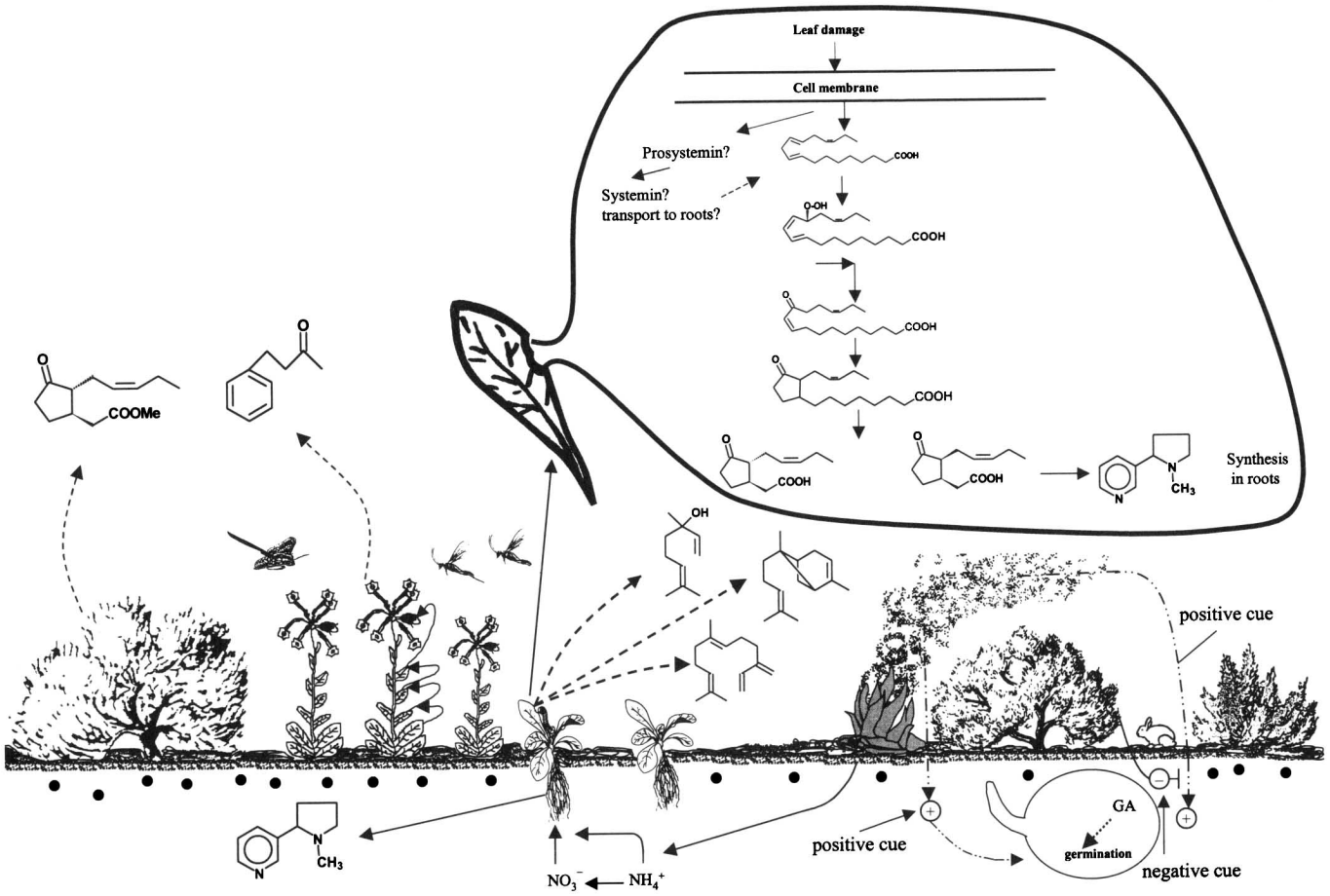
Leaf damage
Cell membrane
Prosystemin?
Systemin?
transport to roots?
COOH
O-OH
Synthesis
in roots
COOMe
OH
positive cue
positive cue
GA
germination
negative cue
NO_3^-
NH_4^+

FIG. 2. The natural history of inducible nicotine production in *Nicotiana attenuata*. The plant is an herbaceous annual that times its germination from a long-lived seed bank in response to positive germination signals (+) produced by the combustion of cellulose during wildfires in sage–juniper habitats of the Great Basin desert and the pyrolytic removal of germination inhibitors (–) (negative cues) that leach from the accumulated litter in the A_0 horizon under the dominant vegetation. The regulation of germination can be overcome by exogenous gibberellin additions, suggesting that the positive and negative germination signals stimulate and inhibit endogenous gibberellin synthesis, respectively. When rosette-stage plants are wounded or attacked by herbivores (rabbits or larvae of *Manduca* spp.), the endogenous wound signal cascade producing jasmonic acid (JA) is activated in wounded tissues, increasing JA pools in shoots, which either directly through transport or indirectly through a signal such as systemin increases JA pools in roots. These, in turn, stimulate nicotine synthesis in the roots and increase nicotine pools throughout the plant. Nicotine is preferentially synthesized from NH_4, which is the most abundant form of nitrogen available in the soil immediately after a fire, and this preference may reduce the biosynthetic costs of nicotine production. As plants reach reproductive maturity, their ability to induce *de novo* nicotine synthesis wanes, but they reallocate previously synthesized nicotine from older leaves to younger leaves and reproductive parts in response to wounding. The use of the jasmonate cascade to activate costly defense responses leaves the plants potentially vulnerable to the emission by nearby *Artemesia tridentata* of volatile methyl jasmonate. When larvae of *M. sexta* attack plants or their oral secretions are applied to leaves, the jasmonate cascade is dramatically amplified and attacked leaves release linalool, ocimene (not shown), bergamotene, and farnesene, which may function to increase the apparency of the attacking herbivore to its parasitoids. The plants increase their apparency to nocturnal pollinators by releasing benzyl acetone from the outer lips of their corolla and withdrawing the nicotine into the base of their corolla below the nectar rewards and the attachment of the filaments. In short, the use of a highly toxic, nitrogen-intensive toxin has a number of consequences for numerous other aspects of the plant's natural history.

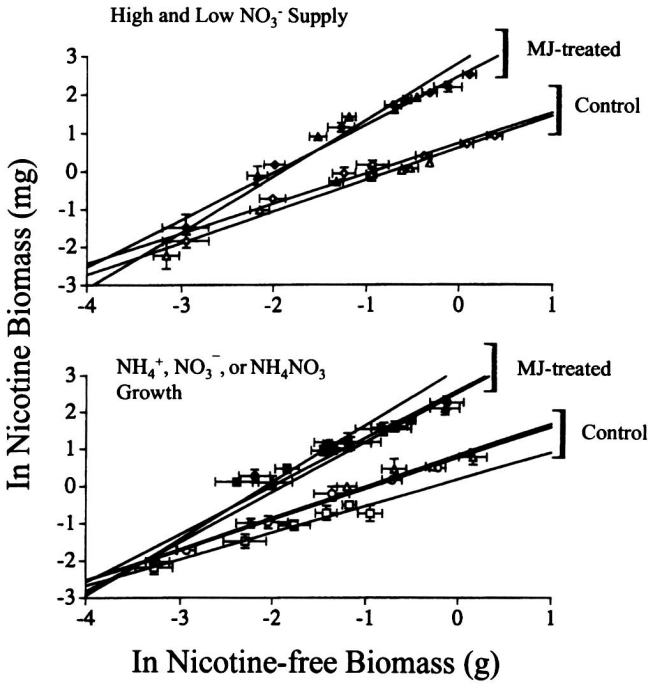

FIG. 3. Whole-plant (WP) nicotine allometries (regressions of ln WP nicotine versus ln WP nicotine-free biomass from many harvests) for control and methyl-jasmonate (MJ)-treated, hydroponically grown plants in an experiment (top) in which plants were grown with high (diamonds) or low (triangles) nitrate supply rates simulating the range of supply rates found in early and late postfire succession and (bottom) from an experiment where plants were grown with ammonium (squares), nitrate (triangles), or ammonium nitrate (circles) at the same nitrogen supply rate. Plants were treated with MJ (filled symbols) to induce nicotine production or not treated (open symbols). Data from (Lynds and Baldwin, 1998). *N. attenuata* has allometrically determined setpoints that respond to the herbivory and endogeneous wound signals but are not influenced by environmental factors that influence plant growth rates.

1998), we examined the effects of different ammonia–nitrate ratios under constant nitrogen supply rates on a plant's ability to accumulate nicotine at induced or constitutive rates of production (Figure 3, bottom). Interestingly, while the nitrogen source and supply had dramatic effects on the rate of plant growth, the allometrically determined rates of nicotine accumulation were not altered. *Nicotiana* plants are remarkably homeostatic in their patterns of allocation to nicotine production and growth, only altering these patterns in response to wounding and the resulting internal signals, not to nitrogen availability.

Because allometric analysis allows one to compare a trait in organisms of different ages and stages, we used it to explore the existence of "immunological memory" in the induced response (Baldwin and Schmelz, 1996). By stimulating the roots of hydroponically grown *N. sylvestris* plants with equivalent quantities of MeJA at different times during rosette-stage growth and allowing plants to completely relax to prestimulated rates of *de novo* nicotine production, we found that plants attained their induced allometrically determined setpoint faster if they had experienced an earlier stimulation. Hence, plants retain a memory of previous elicitations, which allows them to more rapidly attain a particular setpoint; however, the magnitude of the setpoint appears to remain unchanged.

The allometric analysis also provides insights into the changes in the induced response that occur during development and a underscores that the setpoints are not simply determined by the amount of nicotine synthesized in the roots. In the memory experiment (Baldwin and Schmelz, 1996), the magnitude of the setpoints was determined by the number of stimulations rather than by the quantity of nicotine synthesized during a particular stimulation, which varied dramatically during different developmental stages. The allometric slopes of uninduced rosette-stage plants tend to be slightly less than unity, which means that whole-plant nicotine concentrations tend to decrease with growth (Ohnmeiss and Baldwin, 1994). This decrease in whole-plant nicotine concentrations as plants mature reflects the increasing proportion of older leaves on a plant, that older leaves tend to have lower nicotine concentrations than younger leaves (Ohnmeiss et al., 1997), and a waning in the rate of whole-plant nicotine synthesis. As *N. sylvestris* plants begin to flower, they lose their ability to increase *de novo* nicotine production in response to leaf wounding (T. E. Ohnmeiss and I. T. Baldwin, unpublished results) but in response to wounding can redistribute nicotine they already have. This redistribution occurs principally toward reproductive parts and young leaves. Diminished inducibility is neither due to the decreased ability of wounded leaves to produce JA after wounding nor to a decreased ability of roots to respond with increased nicotine synthesis after exposure to jasmonates (T. E. Ohnmeiss and I. T. Baldwin, unpublished results). Rather, the lack of response appears to be due to a decreased ability to transport the wound signal from the leaf to the roots. Because reproductive tissues are strong sinks, which "attract" photoassimilates from source leaves, the sink strength of roots wanes as plants flower, and the altered whole-plant source–sink relationships over ontogeny may be responsible for the waning of inducible nicotine production during flowering. Moreover, such an alteration may also explain the lack of inducibility in plants that have become pot-bound (Baldwin, 1988b) and are no longer strongly exporting photoassimilates from leaves to roots.

Some researchers have argued that the accumulation of nitrogen-containing metabolites, such as nicotine, in response to stress was a means of avoiding ammonia (Rabe, 1990 and refs therein) or amino acid toxicity (Nowacki et al., 1976).

Such arguments are consistent with the application of C/N theory (Bryant et al., 1983; Tuomi et al., 1984; Bazzaz et al., 1987) to the production of nitrogen-containing metabolites. The very existence of homeostatic setpoints that are maintained over a range of growth rates and fertilizaiton regimes undermines the notion that alkaloid accumulation results from an unregulated overflow or waste-product metabolism (Haslam, 1986; Baldwin, 1993). Yet, how this homeostatis comes about remains unclear. Since the sites of nicotine synthesis and accumulation are located in widely separated tissues, simple negative feedback control over nicotine biosynthesis is probably not the responsible mechanism. That these setpoints are not altered when plants acquire nicotine from the hydroponic medium suggests that they may be influenced by nicotine biosynthesis. However, the quantity of nicotine synthesized during an induction is not the only determinant of the nicotine pool at the end of vegetative growth. The intracellular compartments in which nicotine is stored, whether nicotine is transported along apopolastic or symplastic routes to these storage sites, and the factors that control volatilization are all likely to determine how much nicotine accumulates in the shoot when a given amount is produced in the roots.

The localization of nicotine synthesis in the roots and the need for a long-distance transport pathway for the signal to travel to the roots and nicotine to return to the leaves impinges on a plant's defensive capacity. The distances delay production of the defended phenotype, putting the plant in a vulnerable position, and the transport pathways represent a weak link that an herbivore could short-circuit by girdling the plant. Moreover, because nicotine synthesis is located in a tissue distal to the site of damage and the alkaloid is transported in the xylem stream, the plant's ability to target or direct the defense to tissues other than those directly connected by xylem vasculature is limited. These disadvantages beg the question of the advantages of below-ground nicotine synthesis. The advantage becomes apparent when one examines how well a plant can launch a defensive response to an attack that removes a large proportion of the shoot. Only when plants have 88% of their total leaf area removed is their allometrically corrected ability to increase their whole-plant nicotine pool diminished (Baldwin and Schmelz, 1994). The concentration of the last remaining leaf material continues to increase even when plants have lost more than 90% of their leaf area, notwithstanding the impairment of the plant's overall nicotine-synthesizing ability. If a plant's roots produce a given amount of nicotine per day and the plant's shoot suddenly decreases due to herbivory, the recently synthesized nicotine will be sent to a smaller shoot. Hence, the concentration of nicotine in the shoot tissues will rise even if the total synthesizing ability of the root does not change or even declines. Therefore, sequestration of nicotine synthesis in the roots buffers the induced response, making it insensitive to the plant's current photosynthetic capacity and also suggests that the induced nicotine response evolved in response to selection from folivores.

In summary, for the duration of vegetative growth, inducible production of

nicotine can best be understood on the whole-plant scale as changes in allometrically determined setpoints. The rate at which these setpoints are attained is determined by the cumulative number of elicitations a plant has experienced. Once a plant starts reproductive growth, the principal wound-induced alterations in nicotine concentrations come from the reallocation of previously synthesized nicotine, and these reallocations target tissues with high fitness value such as young leaves and reproductive tissues.

ECOLOGICAL CONSEQUENCES

Here, I review the evidence for the fitness costs and benefits associated with inducible nicotine production and examine the mechanisms that might be responsible for the costs. A principal difficulty with the evidence for both the costs and benefits of the responses is the inability to precisely attribute a fitness effect to the production of nicotine *per se*. We are currently unable to surgically alter this trait independently of the other traits that are altered when plants are wounded or treated with jasmonates. We hope to rectify this inability by developing transformation systems for *N. attenuata*. With antisense expression of nicotine biosynthetic genes (PMT) and genes important in the inducible jasmonate production (e.g., LOX and/or AOS), we hope soon to have the ability to independently manipulate inducible and constitutive nicotine production. Until these tools are in hand, however, we can only use techniques that allow us to determine the fitness consequences of altered nicotine production in the context of other traits that are also altered after wounding and herbivore attack. We have used two approaches to examine the costs: (1) the suppression of induced jasmonate production in wounded leaves, by applying auxin (IAA) directly to wounded leaf tissues, which in turn suppresses the nicotine response, and comparing lifetime seed production of plants with suppressed wound-induced responses to wounded plants; and (2) the use of JA to activate wound-induced responses in unwounded plants and comparing lifetime seed production of these plants with those of untreated control plants. For the fitness experiments, we have calibrated the amount of jasmonate needed to simulate the plant response to wounding and avoid jasmonate toxicity. We have determined the amount required to produce a nicotine response comparable to a standardized mechanical wound and the amount required to induce a change in endogenous levels of free JA equivalent to that elicited by leaf wounding (Baldwin et al., 1997b; Zhang and Baldwin, 1997). To target the nicotine response more precisely and to avoid responses that are leaf-specific, we applied the jasmonates to roots. In order to compare the relative importance of physiological and ecological costs of induction, we induce plants growing in environments of increasing ecological complexity; in hydroponic chambers (Baldwin et al., 1998), pots containing soils from the native

environment (Baldwin et al., 1998; Lynds and Baldwin, 1998), plants growing in field plantations (Baldwin et al., 1990; Baldwin, 1998), and in native populations (Baldwin, 1998).

Empiricial Evidence for Costs and Benefits. The benefits of increased nicotine production are readily seen in laboratory bioassays, where culturing plants in nicotine solutions to attain concentrations comparable to those found in induced plants reduces the growth, development, and consumption of nicotine-tolerant lepidopteran larvae (*Manduca sexta* and *Trichaplusia ni*) (Baldwin, 1988c, 1991). Similarly, plants growing in native populations induced with a root jasmonate treatment and subjected to native herbivores (Baldwin, 1998) realize fitness benefits. When leaves from induced plants and adjacent control plants are fed to grasshopper nymphs (*Trimerotropis pallidipennis*), nymphs remove 10 times more leaf area from the leaves of control plants than from those of jasmonate-treated plants (Baldwin, 1998). The benefits are also clearly visible in plants growing in native populations subjected to natural herbivore populations. For example, in one population of 300 pairs of jasmonate-induced and control plants growing in a 2-year-old burn with a high herbivore load, 33% of the control plants lost more than 40% of their leaf area to herbivores, compared to 1% for jasmonate-treated plants. Similar patterns were seen in populations that experienced more modest rates of herbivory; in these populations a fitness benefit could be detected in the lifetime viable seed production of plants. For 300 pairs of plants growing in a 1-year-old burn, 241 pairs (80%) survived to produce seed; of these surviving pairs, jasmonate-treated plants matured on average 11% more viable seed (six capsules or a 349 viable seed/plant benefit of jasmonate treatment) than their untreated counterparts. The fitness benefit varied according to whether the control or the treated member of the pair was more heavily attacked by herbivores. For example, in 160 pairs, both members lost 20% or more of their leaf area to herbivores; in this subset of attacked plant pairs, jasmonate-treated plants matured on average eight more capsules and 537 more viable seeds (a 16–18% benefit) than their control counterparts. In 25 pairs, only the control member of the pair was attacked, and in these pairs, the jasmonate-treated plants matrued on average 35 more capsules and 2848 more viable seeds than controls (a 58–60% benefit) (Baldwin, 1998). In short, plants growing in populations with herbivores appear to realize a fitness benefit from jasmonate-induced responses. While the experiment demonstrates fitness benefits of jasmonate-induction, it does not identify which jasmonate-inducible response(s) effects the fitness differences; we know only that nicotine induction is a component of the response. However, treatment of plant roots should help limit the response to root-specific defense responses.

In the same field experiment (Baldwin, 1998), jasmonate-induced responses were also found to have significant fitness costs if plants are protected from herbivore attack by fencing and insecticide sprays. Jasmonate treatment significantly reduced lifetime viable seed production by 26% (9.9 capsules/plant or

1550 viable seeds) in these protected plants. A similar fitness cost was observed between induced and uninduced members of pairs growing in a 1-year-old burn that had largely escaped herbivory. Plants lost less than 5% of their leaf area to herbivores by the end of the growing season. In this population, jasmonate treatment reduced the lifetime capsule production by 17% (Baldwin, 1998). These reductions correspond to the reduction associated with removing half of a plant's leaf area at the rosette-stage of growth, which reduces capsule production by 27% (Baldwin, 1998). These fitness costs are comparable to those found in a glasshouse experiment with *N. attenuata* plants growing in pots containing burned soil from the plants' native habitat. In this experiment, jasmonate treatment reduced lifetime seed production by 43% (Baldwin et al., 1998). The fitness costs of jasmonate induction observed mirror the costs of wound-induction observed in a plantation experiment with the sibling species, *N. sylvestris* (Baldwin et al., 1990). In that experiment, plants were wounded with a standardized mechanical damage technique and had their wound-induced nicotine response suppressed with IAA applications to the wound site—a procedure that inhibits wound-induced jasmonate production in this species (Baldwin et al., 1997b). The lifetime seed production of wounded plants that exhibited the normal wound-induced nicotine response was 32% less than that of similarly wounded plants that had their wound-induced nicotine suppressed with auxin. The similarity between the two estimates of fitness costs supports the contention that the jasmonate treatment simulated the responses to wounding and that either nicotine production alone or in combination with other jasmonate- and wound-induced responses exacts a large cost to seed production.

In summary, when plants are protected or escape from herbivore attack, eliciting wound responses with jasmonates or wounding significantly reduces the number of viable seeds produced at the time of senescence. This fitness cost, however, becomes a net fitness benefit when plants are exposed to herbivores, and the fitness benefits of the responses induced by jasmonates outweight the fitness costs.

Many mechanisms potentially are responsible for the reduction of seed production associated with high nicotine titers in plants growing in native populations. Two general categories of potential costs will be considered: physiological costs that arise solely from processes within a plant and ecological costs that arise from alterations in a plant's interactions with other organisms in its environment. Since jasmonate-induced reductions in seed production observed in plants growing in glasshouse environments with fewer ecological interactions (43%; Baldwin et al., 1998) tend to be larger than those observed in plants growing in fenced plantations in the field (26% reductions) (Baldwin, 1998), it is reasonable to assume that physiological processes are responsible for most of the fitness reductions.

Physiological Costs of Nicotine Production. In experiments in which unin-

duced *N. sylvestris* (I. T. Baldwin and W. Hamilton, unpublished results) or *N. attenuata* (van Dam and Baldwin, 1998) plants are grown in competition in the same hydroponic chamber or soil-containing pot with jasmonate-induced plants, the observed fitness reductions associated with jasmonate induction are much larger than when plants are grown without competition or when plants are grown with another plant similarily induced. In an experiment with *N. sylvestris* plants grown in hydroponic culture, we demonstrated that the fitness reductions were associated with reductions in competitive uptake of nitrate (I. T. Baldwin and W. Hamilton, unpublished results). This apparent reduction in competitive ability associated with the high levels of nicotine production in jasmonate-induced plants could be due to a number of different physiological mechanisms; two have received some attention: the nitrogen costs of nicotine production and the toxicity of nicotine to photosynthetic processes.

The examination of trade-offs between nicotine and seed production in a nitrogen currency is particularly germane for *N. attenuata*. A molecule of nicotine contains two atoms of nitrogen, and when a plant is induced, 6% of its entire nitrogen content resides in nicotine (Baldwin et al., 1998). Because this investment cannot be recouped by metabolism (Baldwin et al., 1994a, 1998; Baldwin and Ohnmeiss, 1994b), the nitrogen remains unavailable for other activities, such as seed production. This investment of nitrogen likely underestimates the actual costs, because it does not include the nitrogen incorporated into the nicotine biosynthetic enzymes or used in the transport and storage of nicotine. An investment of this magnitude will likely reduce seed production, given that nitrogen availability is demonstrably a fitness-limiting resource of these plants (van Dam and Baldwin, 1998; I. T. Baldwin and W. Hamilton, unpublished results).

Such costs are not necessarily inevitable because *N. attenuata* had evolved life history traits that mitigate the fitness consequences of these nitrogen costs (Figure 2). It is an herbaceous annual that is an important but ephemeral component (usually for only three growing seasons) of the postfire annual community in burned sagebrush, blackbrush, and pinyon–juniper forests of the Great Basin desert (Baldwin and Morse, 1994; Baldwin et al., 1994c; Preston and Baldwin, 1999). After fires, it mass-germinates from long-lived seedbanks, and the seeds are largely dormant until exposed to aqueous extracts of wood smoke. By synchronizing its germination with the postfire environment, *N. attenuata* takes advantage of the high-nitrogen soils that characterize such environments (Baldwin and Morse, 1994; Lynds and Baldwin, 1998). The majority of soil nitrogen after a fire exists as NH_4, which is rapidly nitrified to NO_3 during postfire succession. *N. attenuata* preferentially uses NH_4 rather than NO_3 for nicotine production (Lynds and Baldwin, 1998), perhaps because of the lower biosynthetic costs of synthesizing the alkaloid from NH_4 (2.86 g glucose/g nicotine) than from NO_3 (3.62 g glucose/g nicotine) (Gershenzon, 1994). By producing dormant seeds that germinate after fires, this species selects environments with high

soil NH_4 levels, which in turn allow it to defray the opportunity and ecological costs of this resource-intensive chemical defense. This suggests that the selective importance of the nitrogen-based physiological costs will strongly depend on the germination behavior of the seeds (Figure 2).

The use of chemicals for defense inevitably entails coping with the possibility of autotoxicity, for the simple reason that the most effective chemical defenses are likely to be broadly biocidal. Nicotine, for example, is demonstrably toxic to *N. sylvestris* plants although the species clearly lacks an acetylcholine-based nervous system, which is the target of nicotine's toxic effects in herbivores. We quantified the accumulation of and tolerance to exogenously fed nicotine by monitoring photosynthetic capacity and growth in two nicotine producing species (*N. sylvestris* and *N. glauca*) as well as in two solanaceous species (*Datura stramonium* and *Lycopersicon esculentum*) that do not produce nicotine, in order to examine the relationship between tolerance and the ability to produce nicotine in defensive quantities (Baldwin and Callahan, 1993). All species exhibited reductions in the photosynthetic capacity of nicotine-fed plants, and nicotine-producing species showed no greater tolerance as measured by photosynthetic capacity than the two nonproducing species. Wounding marginally increased the tolerance of *N. sylvestris* to exogenously fed nicotine, suggesting that photosynthetic tolerance is coordinated with nicotine production. Whether endogenously produced nicotine causes similar physiological difficulties for a plant remains to be determined. The solution to the problem of autotoxicity lies in the details of the mechanisms responsible for the production, storage, and release of the chemical defenses, and until endogenous production can be manipulated independently of the other wound-induced responses, it will be difficult to determine whether any part of the fitness reductions associated with nicotine production are directly a result of nicotine toxicity.

Ecological Costs of Nicotine Production. The problem of physiological autotoxicity associated with the use of a chemical defense corresponds to the problem of repelling or killing beneficial organisms. For example, well-defended plants may have difficulties attracting pollinators; in this regard, it is interesting to examine how *N. attenuata* alters its nicotine pools in the tissues that interact directly with pollinators, namely the corolla. Nicotine can be detected in the headspace of flowers and is localized principally in the basal parts of the corolla, below the attachment of the filaments and the nectar reward (Euler and Baldwin, 1996). In contrst, benzyl acetone (BA, 4-phenyl-2-butanone; Figure 2) is found only in the outer lip of the corolla, where pollinators come in contact with the flower. BA is released from the corolla in the evening, with emissions reaching maximum values just after dark—a pattern of emission characteristic of moth-pollinated flowers (Euler and Baldwin, 1996). Diurnal changes in the size of the corolla pool closely parallel the amount emitted by flowers throughout the day. Moreover, pollination dramatically decreases both the pools of BA in the

corolla and its emission from flowers. Interestingly, the corolla pools of nicotine are stable throughout the day except during the period of peak BA production and emission when nicotine pools decrease significantly (Euler and Baldwin, 1996). This highly coordinated exchange of a defense metabolite with one that seems to function in pollinator attraction suggests that the evolution of chemical defenses has favored plasticity in order to solve the ecological problems that attend broadly biocidal chemical defenses.

The ecological dilemma highlighted by the interplay of BA and nicotine in the corolla is echoed in another suite of responses with which plants try to increase their "apparency" to other members of the ecological community. Many plants actively release volatiles in response to herbivore attack, and these volatiles appear to function as indirect defenses that guide the foraging behavior of predators and parasitoids (Turlings et al., 1995; Takabayashi and Dicke, 1996; Alborn et al., 1997). Therefore, it is particularly interesting to examine the coordination of these plant alarm cells with the induction of secondary metabolites that are broadly biocidal. Many plant defense metabolites are sequestered by a plant's herbivores and used against the predators of the herbivores—turning a plant defense against itself. Plant-produced pyrrolizidine alkaloids (Eisner and Meinwald, 1995; Rowell-Rathier and Pasteels, 1992), cardenolides (Malcolm and Zalucki, 1996), iridoid glycosides (Bowers, 1988), and salicylates (Smiley et al., 1985) are actively sequestered by adapted insects and used for their own defense. A handful of insects are tolerant of nicotine's toxic effects and are capable of feeding on plants containing high concentrations of nicotine. Rapid excretion of the toxin in combination with target-site insensitivity appear to be the main mechanisms of resistance for these adapted herbivores (Self and Guthrie, 1964; Parr and Thurston, 1972; Krischik et al., 1988; Murray et al., 1994; Snyder and Glendinning, 1996). Whether or not herbivores actively sequester nicotine for defensive purposes is unknown. However, since the amount of plant material in a caterpillar gut can account for half of its weight (Rowell-Rathier and Pasteels, 1992), and defense metabolites are frequently found in the hemolymph, active sequestration of nicotine is not necessary to provide a defense against predators or parasitoids. For example, the tobacco hornworm *Manduca sexta* does not actively sequester nicotine, but its hemolymph retains quantities of the toxin sufficient to cause significant mortality of its parasitoid wasp, *Cotesia congregata* (Barbosa et al., 1991). Thus, although nicotine is not one of the sequestered secondary metabolites, it may wreak havoc with the plant's ability to use top-down processes as a defense.

We examined the responses of both *Nicotiana* species to the feeding damage of the nicotine-adapted herbivore, *M. sexta*, and compared these responses to mechanical simulations of the feeding damage. These comparisons reveal that plants respond differently to an herbivore than to the damage the herbivore causes. These comparisons demonstrate that larval feeding suppresses the nico-

tine response induced by a comparable amount of mechanical wounding (Baldwin, 1988c; McCloud and Baldwin, 1997) and that it dramatically amplifies the induced JA production many times more than the amount induced by mechanical wounding (McCloud and Baldwin, 1997). Moreover, the application of oral secretions from the larvae to a mechanical wound results in both the amplification of the wound-induced JA response and the suppression of the nicotine response (McCloud and Baldwin, 1997). At first glace, these adapted larvae appear to be feeding in a "stealthy" fashion, reducing the nicotine responses below that of plants suffering a comparable amount of tissue loss from the same portions of their canopy over the same time period and uncoupling the relationship between the endogenous wound signal, JA, and the response to this wound signal (Ohnmeiss et al., 1997; Baldwin, 1998); but, upon closer analysis, it seems to be the plant that is optimizing its defense response to this nicotine-tolerant herbivore. This conclusion is suggested by an additional plant response elicited by the feeding of this herbivore.

Larval feeding and the application of larval oral secretions or jasmonates to damaged leaves result in a dramatic increase in the release of both monoterpenes (ocimene and linalool) and sesquiterpenes (bergamotene and farnesene; Figure 2), which are not released after only mechanical wounding (J. Kahl, C. Preston, R. Aerts, R. Halitschke, A. Lorenz, and I. T. Baldwin, unpublished results). When these same volatile compounds are also released by corn and cotton in response to herbivore attack, they are thought to function as alarm calls, attracting parasitoids (Turlings et al., 1990; Takabayashi and Dicke, 1996; DeMoraes et al., 1998), and they may be playing a similar role in *Nicotiana*. The plant may be actively suppressing its nicotine response, thus avoiding undermining the use of the third trophic level as a defense against this relatively nicotine-tolerant herbivore (Krischik et al., 1988). The use of indirect defenses instead of direct defenses is particularly advantageous when the plant is being attacked by a nicotine-tolerant herbivore or when the nicotine response is impaired [such as when plants are pot-bound (Baldwin, 1988b) or are likewise restricted in their root growth], or if the fitness costs of nicotine production are too high as a result of nitrogen limitations.

The actual fitness consequences of using different plastic responses will be determined by the particular ecological milieu that the plant finds itself in and, therefore, requires an extensive amount of experimental fieldwork manipulating volatile emissions and nicotine accumulations independently of one another. Moreover, the manipulations to examine the fitness consequences of the plastic responses should be performed in a number of different populations to establish the generality of the fitness effects. It is difficult to predict the fitness consequences of plant traits that increase apparency to other members of the community. Volatiles emitted from plants could readily be detected by the herbivores in addition to parasitoids and predators and increase rates of attack. For example,

when *N. attenuata* plants growing in natural populations were fitted with devices that increase the amount of BA emitted from individual plants by approximately an order of magnitude, they lost more tissue to herbivores than did unmanipulated controls (Baldwin et al., 1997a). Hence, when the emission of a compound thought to be important in pollinator attraction was increased and made constitutive, the plants incurred a higher herbivore load. In short, *Nicotiana* plants have both direct and indirect defenses at their disposal, and the efficacy of these different types of defense varies enormously depending on the particular ecological situation in which the plant finds itself.

CONCLUSIONS

Herbivore attack on *Nicotiana* plants elicits a battery of plastic responses, and nicotine induction is only one of the many direct and indirect defenses that are induced. These defense responses, in turn, are embedded in a physiological reconfiguration of growth-related processes that may provide the necessary adjustments of metabolism to make the defense responses possible or to increase the tolerance of plants to the fitness consequences of tissue loss to herbivores. For example, in *N. sylvestris*, wound-induced nicotine production is coordinated with an increase in photosynthetic rates in undamaged leaves (Baldwin and Ohnmeiss, 1994a). This increase may help compensate for part of the physiological costs of nicotine production and also increase a plant's tolerance to the physiological stress of leaf loss.

Some of these responses are activated by the jasmonate cascade, and the activation of this cascade results in substantial fitness reductions when plants are growing in environments protected from herbivores. Although jasmonate-induced responses are clearly costly, it is not clear that the fitness costs reflect the costs of a defense response *per se* (e.g., nicotine production, volatile emission, etc.). In other words, it is not clear that it is the part of the response that functions defensively that is costly; the fitness costs may result from reductions in growth, which may in turn be an adaptive response to herbivore attack if, for example, slower growing plants are less apparent to herbivores or more tolerant of the fitness consequences of tissue loss to herbivores. Determining which of the many jasmonate-induced processes are costly is important if we want to draw conclusions about the processes that maintain variations in constitutive resistance in natural populations. At this point, it is not clear whether our data demonstrating a large cost of jasmonate-induced resistance apply to the evolutionary questions of the costs of defense or the costs of tolerance (Rausher, 1996) and whether these costs can be diminished by selection (Simms, 1992; Karban and Baldwin, 1997). We clearly need to dissect the responses elicited by this "master switch" to determine the fitness consequences of each component of the response.

While the analysis of the physiological costs will be limited by tools that are available to manipulate components of the response independently, the analysis of the ecological costs will additionally be limited by our understanding of the plant's natural history. Only by understanding how a plant's predators, competitors, and mutualists respond to the different phenotypes a given genotype can produce will we understand the ecological costs of plasticity. For example, although plants appear to have almost unlimited possibilities in being able to specifically tailor the responses elicited by the jasmonate cascade to particular biotic agents (Baldwin, 1999), the fact that this cascade is found in most higher plants (Creelman and Mullet, 1997) may prove to be a liability since competitors or predators could manipulate it for their own benefit. The production of volatile methyl jasmonate in apparently physiologically active quantities by *Artemesia tridentata* foliage (Farmer and Ryan, 1990) and the production of coronatin, a phytotoxin that is a structural mimic of the jasmonate biosynthetic precursor, 12-oxo-phytodionoic acid (Blechert et al., 1997; Weiler, 1997) by some *Pseudomonas syringae* pathogen varieties (Bender and Palmer, 1996) may be examples of this liability.

The ecological costs of plasticity that relate to the reliability and information content of the signals that plants use to alter their phenotypes represent the most difficult empirical challenge for researchers testing the cost–benefit model. If the signals do not reliably predict the occurrence of an environment, then a plant will have a high probability of finding itself with the "wrong" phenotype for a particular environment. A comparison of fitness costs of different phenotypes in different environments and the probability of a mismatch occurring represent important constraints to the evolution of plastic responses. However, assessing the fitness consequences of mismatches requires an intimate understanding of the plant's natural history to appreciate how they might come about. Again, *N. attenuata*'s plastic responses provide an example of this complexity.

While *N. attenuata*'s inducible responses to herbivore attack are likely to have evolved in environments where the probability of attack is intermediate and selected by the high physiological costs of the responses, as theory would predict (Zangerl and Bazzaz, 1992), the evolutionary response to herbivore attack in this species begins with the ability to time germination with the occurrence of fires. It is only in the immediate postfire environment of the Great Basin desert that *N. attenuata* will reliably find intermediate levels of herbivore attack; by waiting for the postfire environment, this species escapes most of its herbivores (Baldwin, 1998). Moreover, *N. attenuata* reduces the physiological costs of its nitrogen-intensive defense if it coordinates its germination with fires, because it can use the large supply of soil ammonia that occurs after a fire. If *N. attenuata* seeds germinate from their long-lived seed bank into the correct habitat, namely, a recently burned environment, they realize a 2- to 12-fold increase in lifetime seed production as compared to plants germinating in unburned soils (Baldwin et al., 1998; Lynds and Baldwin, 1998; Preston and Baldwin, 1999).

This large fitness benefit of correctly locating the postfire germination niche may explain the use of both positive germination signals from smoke (which can travel far from the burned habitat) as well as the removal of inhibitory germination factors from the litter of mature intact vegetation (Preston and Baldwin, 1999). The combination of both positive and negative control over germination helps focus germination to the appropriate habitats (Preston and Baldwin, 1999) and represents a highly reliable ecological signaling system. In contrast to these large fitness costs, the costs of jasmonate induction are relatively small (20–40% reduction in lifetime seed set of producing a defended phenotype in herbivore-free environments), suggesting that selection for accurate germination signals will be much stronger than for accurate wound-signals and perhaps also select for linkages between germination and defense responses. Given the complexity of the mechanisms coordinating these plastic responses and the complexity of evaluating the costs and benefits of their consequences, the study of phenotypic plasticity demands the expertise of chemists, ecologists, molecular biologists, and evolutionary biologists: in short, the training of a chemical ecologist.

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FROM CHEMICAL TO POPULATION ECOLOGY: INFOCHEMICAL USE IN AN EVOLUTIONARY CONTEXT[1]

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Abstract—The marriage of chemistry with ecology has been a productive one, providing a wealth of examples of how chemicals play important roles in the loves and lives of living organisms. At first the marriage may have been a simple and monogamous one with the major scientific aim of making proximate analyses of chemically mediated, individual level interactions. But times have changed and chemical ecology is broadening, embracing different approaches and disciplines. There is, for example, increasing appreciation of variability in the systems under study and an increase in evolutionary thinking. Another promising development is greater recognition of the potential importance of chemically mediated interactions for population dynamics and for structuring communities and species coexistence. The latter is an utterly underexplored area in chemical ecology. The field of chemical ecology of insect parasitoids shows some of these promising developments. Responses of parasitoids to infochemicals are increasingly studied with an integrated approach of mechanism and function. This integration of "how" and "why" questions significantly enhances the evolutionary and ecological understanding of stimulus–response patterns. The future challenge in chemical ecology is to demonstrate how chemically mediated interactions steer ecological and evolutionary processes at all levels of ecological organization. To reach this goal there is a need for interdisciplinary collaboration among chemists and ecologists working at different levels of organization and with different approaches, with other disciplines as partners.

Key Words—Chemical ecology, evolution, variation, population dynamics, community, species interactions, infochemical, semiochemical, parasitoid, foraging behavior, learning, phenotypic plasticity.

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1. INTRODUCTION

The objects and approaches in ecology are diverse. Hard-core ecology has the ambitious aim to understand how organisms on this earth manage to maintain themselves, and from this how populations, communities, and ecosystems function. The large-scale destruction of natural habitats and the frightening massive extinction of species urge us to seek fundamental insight into the processes that affect the abundance and distribution of organisms. For evolutionary ecologists, who try to interpret the structure and function of organisms, communities, and ecosystems in the context of evolutionary theory, this means elucidating the selection pressures that shape characteristics at these different levels of ecological organization. The selection pressures are often related to competition, to the distribution and exploitation of resources, and to how niches are differentiated. Many of these processes are dependent on the characteristics and functioning of individuals, for organisms are, after all, the fundamental units of ecology. Since chemicals play an important role in interactions of just about every living creature on this earth, the role of chemicals in steering all kinds of ecological and evolutionary processes must be extensive. It should, therefore, be a challenge for chemical ecologists to show experimentally the functional role of chemically mediated interactions at the different levels of ecological organization.

Chemical ecology should be an interdisciplinary research field where chemists and ecologists combine their different perspectives to obtain a greater understanding of ecological and evolutionary processes. Chemical ecology had a traditional focus on proximate analysis of chemically mediated individual level interactions (Jones, 1988) (Figure 1). Studies generally involved chemical isola-

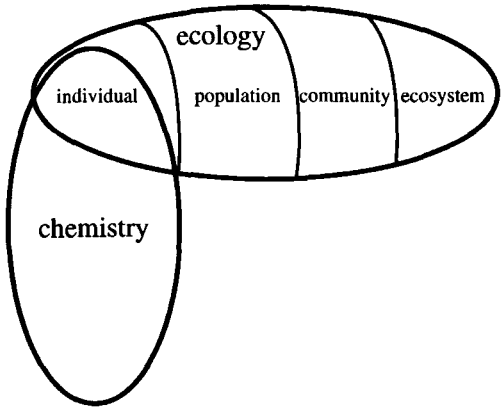

FIG. 1. In the past, the field of chemical ecology covered mainly individual-level interactions.

tion, characterization, and synthesis of chemicals combined with physiology and behavior. Over the years, chemical ecology has produced a wealth of examples of how chemicals play important roles in the loves and lives of living organisms, both plants and animals, vertebrates and invertebrates. There was traditional emphasis on insect chemical communication, and Silverstein and Simeone were important pioneers in this successful research area through their individual research (e.g., Silverstein, 1990) and as editors of the *Journal of Chemical Ecology*.

VARIATION: BOON OR BAIN, PAIN OR PLEASURE?

The concept of self-replication is unique to biology, and the phenomenon of variation through mutation and recombination, multiplication, and evolution through natural selection dominates present-day biological thinking. Variation is intrinsic to living organisms. Each individual has unique characteristics leading to variation at higher levels of ecological organization. In addition to genetic variation between individuals, there is variation in the expression of genotypes in different environments. Furthermore, there is within-individual variation as a function of physiological state (age, hunger, mating condition), experience, and external biotic and abiotic environmental conditions. Appreciation of this variation between and within individuals and among populations is essential for evolutionary ecologists (however, the variation between individuals is often obscured in traditional ecological studies and theory (Uchmánski and Grimm, 1996). In this respect, there is quite a "culture difference" between ecologists and chemists. For hard-core chemists, variation has a different meaning. In chemistry, as in physics, processes are deterministic and variation can generally be attributed to measuring techniques, not to intrinsic characteristics of the system under study. Physicists and chemists may be worried or annoyed by variation, while ecologists are intrigued by it. Chemists working in the field of chemical ecology do encounter biological variation since the sources of the chemicals under study are living organisms.

Variation thinking lies at the basis of evolutionary "selection thinking," i.e., that differences in phenotypes can be correlated to variation in fitness, setting the stage for natural selection. "Nothing in biology makes sense except in the light of evolution," to use the famous words of Dobzhansky. Although there may be a theory of inorganic evolution, derived from the Darwinian evolutionary theory, that finds application in the exact sciences in models on the origin of the elements, the planets, and the universe, the field of chemistry has little affinity with the concept of natural selection and evolution. Again, this is different for chemists that work in the field of chemical ecology where variable living systems are being investigated. Indeed, present-day chemical ecologists are increasingly

aware of aspects of both genetic and environmentally induced variation (Roitberg and Isman, 1992; Cardé and Bell, 1995; Schoonhoven et al., 1998). It is interesting to note that different fields have different foci on variation. The area of insect sex pheromone communication is more concerned with genetic variation (e.g., Löfstedt, 1990, 1993) than with environmentally induced variation (but see McNeil, 1991). In contrast, in the field of plant–insect interactions and parasitoid ecology, there is a strong emphasis on phenotypic variation (e.g., through learning) (e.g., Jaenike and Papaj, 1992; Vet et al., 1995). This may not be so surprising and may in fact reflect biologically realistic differences between systems in the importance of these two sources of variation. Stabilizing selection pressures, acting on coevolving signal–receiver systems, such as the chemical communication between sexes, may have reduced the benefit and likelihood of phenotypic variation. This is different for insects that respond to diverse and variable chemical sources such as plant volatiles. Here the signal–noise ratios are of a different dimension, and there is greater selection on flexibility in the response of individual insects. Hence, congenitally fixed responses to such cues are less likely to evolve (Vet et al., 1990).

With respect to an evolutionary approach, there are also historical differences between some areas of chemical ecology. Research on plant–herbivore interactions, and especially plant defense, has always had a strong emphasis on evolution (e.g., Feeny, 1976; Futuyma, 1986; Rausher, 1992; Berenbaum, 1995; Karban and Baldwin, 1997). As mentioned above, in the area of insect pheromones, evolutionary studies focus on genetic variation between individuals and populations, while the recognition of other aspects of variation is more recent (Bell et al., 1995).

There is also a difference between some fields in the way evolutionary theory is developed. The approach in the field of plant defense is very much one of starting with and emphasizing the details of the natural history of the system in order to study its evolution. This system-specific approach may have constrained the development of general theory (Price, 1991; see Berenbaum, 1995, for a discussion). This approach seems different from that of evolutionary ecologists studying parasitoid–host interaciton where the emphasis has been on the development and testing of general theory (Godfray, 1994).

INTEGRATED APPROACH

Chemical ecology aims to enhance the ecological and evolutionary understanding of interactions where chemistry plays an important role. Several authors have stressed the importance of an integrated approach to reach this goal, combining the different approaches of both chemists and ecologists (e.g., Roitberg, 1992; McNeil, 1991, 1992; McNeil et al., 1997; Phelan, 1997). Roitberg (1992),

for example, referred to "the four questions" of the Dutch ethologist and Nobel Prize winner Tinbergen (1963). When studying a biological phenomenon, consideration should be given to questions on the function (the ultimate "why" questions), causation (the proximate "how" question), phylogeny (evolutionary history, lineage), and ontogeny (development) of the phenomenon. Although most easily applicable to behavioral studies, this integrated approach has value for the study of any biological phenomenon. Combining the different perspectives and approaches of chemists and ecologists and studying both proximate and ultimate factors in concert, and, where possible, including aspects of lineage and development, increases the chance that generalizations about systems can be made and patterns detected. After all, this is the ultimate goal of the study of ecology.

In addition to the integrated approach, there is another promising development in chemical ecology, i.e., the expansion from individual level to population level processes. This expansion requires true interdisciplinary collaboration between individual-oriented researchers such as behaviorists, physiologists, and chemists with theoretical and experimental population ecologists. Ecology would benefit from a greater insight into how chemically mediated interactions can influence the spatial and temporal dynamics of the populations involved. It will be a challenge to assess the direction and dimension of this influence. And, of course, pushing it further would mean expanding towards the community level by looking at how chemically mediated interactions can shape the coexistence of species, an area that is utterly unexplored in chemical ecology.

CHEMICAL ECOLOGY OF PARASITOID FORAGING IN AN EVOLUTIONARY CONTEXT

We can see some of these developments in the field of chemical ecology of insect parasitoids. I will use examples from this field to clarify the points I have raised. The examples reflect my personal interest, i.e., variation in behavior of individual insect parasitoids, where chemicals play an important role in decision making. Parasitoids live in a complex chemical world. Their major task is to find other insects in or on which to lay their eggs. Chemical cues are a major source of information in this searching process, which has intrigued chemical ecologists for years (Lewis et al., 1976; Vinson, 1984; Vet and Dicke, 1992; Turlings et al., 1993). For an excellent and comprehensive review on the behavioral and evolutionary ecology of parasitoids, I refer to Godfray (1994).

Before the 1980s there was a descriptive and mechanistic approach to the process of host searching in insect parasitoids. Chemical ecologists described the steps of how a parasitoid finds its host and identified the infochemicals involved. These infochemicals come from the host itself or its food and/or from other organisms associated with the host. The focus was on specifying

stimulus–response sequences, and the behavioral outcome was conveniently presumed to be constant. For each system, novel cues had to be identified and generalizations over systems were hard to make. Since the 1980s, more and more research questions arose from the field, novel bioassays were developed, and the studies shifted from short-range searching, where host cues are important, to longer range searching, where the food of the host (often plants) plays a major role. This is where behavioral variability entered the scene. The study of plant–parasitoid interactions revealed great variability both in the responses of the animals and in the stimuli involved, the plant volatiles.

General patterns of selective forces were recognized that shaped the use of infochemicals. By using an evolutionary approach, questions were asked like: can we understand why some stimuli (e.g., host or plant-derived cues) are more important than others, and can we see patterns in this over species? Why are responses to some cues invariably high, and responses to others highly variable? What is the function of behavioral plasticity? Is behavioral plasticity related to ecological characteristics, such as the diet breadth of the species involved or the host stages they attack (Vet and Dicke, 1992; Vet et al., 1995)?

Behavioral Variation Through Learning. Like most animals, foraging parasitoids have to deal with temporal or spatial changes in resources. In a changing environment, flexibility in behavioral response is a necessity and learning provides an adaptive mechanism (Papaj, 1993b). Parasitoids emerge as inexperienced animals with a set of responses to major stimuli that they use to locate a host (Vet et al., 1990). However, this initial response profile has limited value when the environment changes over and between generations. Through learning, parasitoids can acquire novel responses or enhance existing responses to stimuli that appear to be reliable indicators of host presence during the parasitoid's foraging life (see Turlings et al., 1993; Vet et al., 1995, for reviews). Experiments with several parasitoid species show they learn to respond to stimuli after reinforcement with host encounters, but unrewarding experiences also can play a significant role in determining whether parasitoids respond to a stimulus or not, or which stimulus is preferred in choice situations (e.g., Papaj et al., 1994; Vet et al., 1998). Hence, parasitoids not only base their foraging decisions on external information, e.g., the chemicals they encounter, but also on internal information, i.e., experience-based information, stored in their memory. Integration of these two sources of information leads to behavioral performance. Chemical ecologists who ignore the internal component of this integration are missing half the fun! What's more, they are neglecting a major source of variation in behavior that can have significant meaning for population processes.

Behavioral Ecology Approach. A behavioral ecology approach to parasitoid chemical ecology, where the focus is on adaptive decision making by the animal, would be to ask how variation in responses to chemical cues affects the animals' fitness. We need insight into the following aspects: (1) What is the

"currency" the animal tries to maximize? The direct link between the number of hosts parasitized and the production of offspring generally makes host encounter rate a suitable currency, especially for animals that are limited in the time available for foraging. Selection will favor animals with the highest life time host encounter rate. (2) What are the animal's choices, i.e., what is the variation in resources (and chemical cues related to this variation) that the animal is going to perceive under natural conditions? It is obvious that "knowing your system" is of great importance for researchers to provide the answers to this question. (3) What is the range of behavioral possibilities the animal has (to respond in a certain way, motor pattern, attraction, avoidance etc.). (4) What are the constraints involved (e.g., sensory perception, memory capacity, as well as time available for foraging, etc.)? Answers to these questions are essential if we want to construct optimization or other evolutionary models to make actual quantitative predictions about animal behavior (Stephens and Krebs, 1986). Obviously, not all aspects can be known in detail. Behavioral experiments can provide answers to the third and, to a certain extent, the fourth question, but assumptions still have to be made. Predictions can then be made and experimentally tested on how animals should respond to chemical cues in a variety of situations. This way we can assess the correlation between variation in behavioral responses to chemicals and variation in fitness, providing us ultimate insight in the use of chemical information. Returning to the four Tinbergen questions, the question of lineage can, to a certain extent, be brought in by comparing the searching behavior of phylogenetically and ecologically related species. Relationships between the degree of phylogenetic relationship and the degree of similarity in searching behavior can help to assess historical constraints and provide insight into adaptive radiation and convergence of behavioral traits (Vet and van Alphen, 1985). The question on ontogeny relates to how responses of adults are modified during larval development (e.g., on a certain host or host plant). For insect parasitoid foraging behavior, this preemergence experience has been shown to play a less significant role than experience during the adult stage, which strongly modifies responses to chemical foraging cues (Turlings et al., 1993; Vet et al., 1995).

Function of Behavioral Variation. Can we demonstrate that experience-induced variation in the way parasitoids respond to chemical cues is functional? Papaj and Vet (1990) showed a reduction in travel time (time it took to find a host food substrate) through learning, in field experiments with *Leptopilina heterotoma*, a parasitoid of drosophilid fly larvae that feeds in decaying or fermenting plant or mushroom substrates. Parasitoids are attracted to the odors of these substrates. Learning not only enhanced the rate of substrate location but also influenced the choice of substrate. Vet and Papaj (1992) quantified precisely how learning affected movement in a substrate odor plume. With a Kramer-type locomotion compensator or "servosphere" it was shown that female *L. heterotoma* walked faster and straighter, made narrower turns, and spent more time

in upwind movement (towards the odor source) in the odor from the substrate with which they had experience than in odor from another type of substrate. In addition to affecting the mean of these behavioral parameters, experience also reduces the variability around those means, and simulation models (Papaj, 1993a) strongly suggest that this consistency facilitates discovery of the substrates, as observed in the field (Papaj and Vet, 1990).

Sampling. Regarding questions 2, 3, and 4 raised above, we can ask: do parasitoids make functional choices when confronted with variation in chemical information? In most optimal foraging models, animals are considered to have complete information about the profitability of the current and other patches in the habitat, which enables them to make optimal foraging decisions. This assumption of omniscience has often been criticized as biologically unrealistic. It is more realistic to assume that, just like any foraging animal, searching parasitoids face a problem of incomplete information. Hence, parasitoids also need to sample, and experience plays a major role, both in patch selection (where to go) and patch exploitation (how long to stay and which hosts to accept). The importance of experience is indeed assumed in some more realistic patch models and has been shown empirically (e.g., Bernstein et al., 1988, 1991; Haccou et al., 1991; Hemerik et al., 1993).

Several studies indicate that sampling may indeed play a significant role in decisions that parasitoids make on the basis of chemical information. *Cotesia glomerata*, a parasitoid of *Pieris* caterpillars, is capable of assessing patch profitability on the basis of olfactory (plant-derived) volatile cues, and females use experienced host density as a cue to establish a preference for plant species (Geervliet et al., 1998). Although this shows that parasitoids can use and process information relating to the assessment of resource availability, there is still a lack of insight as to how parasitoids (and insects in general) do so. It is unclear, for example, how insects distinguish between noise and relevant variation in stimuli, and this has consequences for interpreting how animals use chemical cues.

A Functional Approach to Stimulus Discrimination. In a sense, this question of how insects distinguish between signal and noise pertains to the subject of stimulus discrimination, an area generally studied from a mechanistic, psychological, or sensory physiological perspective. Adding the behavioral ecology approach enhances our insight into the choices animals make. We can ask: when would it benefit the animal to discriminate between stimuli (in terms of maximization of its host encounter rate)? Using the parasitoid *L. heterotoma*, we studied the function of learning to discriminate between odor stimuli (Vet et al., 1998). We hypothesized that decisions to consider variation in odors and to discriminate between them, or to ignore variation, are based on the completeness of information the animal has about differences in substrate profitability. In wind-tunnel studies, females learned to discriminate between odors from substrates that were qualitatively different, for example, between odors from apple and pear substrates or between yeast substrates with different C_6 compounds added. They did not discriminate when dif-

ferences were small (e.g., between odors from two apple varieties or between yeast substrates with different concentrations of ethyl acetate), unless previously unrewarding experiences provided evidence of the absence of hosts in one of the substrates. Hence, these results suggest that nondiscrimination between odor stimuli in this species is not a lack of ability to discriminate but a functional choice by the parasitoid. Other studies should test the generality of the hypothesis that discrimination and nondiscrimination of odor stimuli are dynamic behavioral decisions, related to the degree of variation in chemical information from natural resources and to an animal's informational state.

In conclusion, the convenient assumption of omniscience in optimal foraging models may not be so biologically unrealistic for parasitoids that learn fast and make sophisticated use of volatile infochemicals informing them of relevant variation in resource availability. With the use of general learning mechanisms such as associative learning, combined with the sensory and neural ability to perceive and interpret small differences in environmental variation, parasitoids can make adaptive foraging responses on the basis of external (chemical cues) and internal (experience, memory) information. Combining questions on the mechanism and function of behavior significantly adds to our insight in infochemical use by insect parasitoids.

FROM INDIVIDUAL TO POPULATION PROCESSES AND FURTHER . . .

Host Searching Behavior Affects Population Dynamics. The famous ecologist A. J. Nicholson (1933) recognized the searching process as a major factor in population dynamics with the following: "... animals appear to be limited in density, either directly or indirectly, by the difficulty they experience in finding the things they require for existence, or by the ease with which they are found by natural enemies."

Since to find or be found is often a matter of chemicals, this illustrates the importance of chemical ecology for population level processes! Responses to chemical cues determine the rate with which microhabitats, patches of hosts, or individual hosts are encountered. The rates of encounter strongly affect the optimality of other, subsequent foraging decisions that, to a large extent, are also based on responses to chemical cues. Optimality models predict, for example, that an increase in the encounter rate of patches will: (1) decrease the optimal time spent foraging on a patch (that is being depleted due to the act of foraging), (2) reduce the optimal set of patches that should be exploited (start to ignore the bad ones), (3) increase selectivity in host acceptance (no longer accept the less suitable ones), and (4) reduce clutch size (number of eggs) laid per host.

Such effects are not only interesting from an optimality, behavioral ecology perspective, they are important to the underlying principles of biological

control since they have population dynamic consequences. Patch and host selection decisions affect the spatial heterogeneity in attack rates, a significant factor in the persistence of parasitoid-host interactions (Taylor, 1993). An increase in selectivity can create greater refuges for less suitable host species or stages, which has important consequences for population dynamics. The way parasitoids perceive host distributions—whether from a distance with the use of plant cues or through host-derived contact chemicals after encountering a host aggregation—may affect how parasitoids exploit hosts (e.g., whether parasitoid attack is density dependent). Similarly, wheher or not parasitoids can use chemical information to discriminate between small and large host aggregations can affect spatial heterogeneity in attack rates. Predator–prey models by Bernstein et al. (1991) showed that travel costs (let's say time to find patches) were a significant factor in determining predator distribution and prey depletion. Hence, choices by individuals can influence the spatial distribution of host mortality. Inversely, population distributions can also affect the optimality of decision rules in individuals (Driessen et al. 1995; Vet, 1996; Vos et al., 1998).

Competition Avoidance, Niche Differentiation, Species Coexistence. Infochemicals can play a major role in the avoidance of intra- and interspecific competition, important processes affecting the dynamics and spatial distribution of populations and the coexistence of species. Insects are known to mark their hosts after oviposition. The behavioral ecology of host discrimination and the avoidance of super- and multiparasitism by insect parasitoids has been the subject of many theoretical and empirical investigations (e.g., van Alphen and Visser, 1990; Weisser and Houston, 1993) and the evolution of such oviposition marks has been studied by Roitberg and Mangel (1988). Although marking evolved to benefit the marking female, individuals from the same (or another) species "speaking the same language" can take advantage of this chemical information conveyance and avoid competition through host discrimination when survival of the additional egg is low. The behavior we observe is variable since host discrimination is not always adaptive (van Alphen and Visser, 1990). Hence, an observed lack of discrimination is often not an inability of the animal to discriminate but a functional response not to do so.

Considering the chemical ecology of such interactions, most studies concern contact chemical marks. Much less investigated is the role of volatile cues in competition avoidance. However, recent work has shown that parasitoids may use chemical information of potential competitors actively to avoid sites where competitors are present. For example, the generalist *L. heterotoma* uses volatile cues to avoid patches where its superior competitor, the specialist *L. clavipes*, is parasitizing (Janssen et al., 1995). Inclusion of this parameter in a simple patch selection foraging model made predictions of the model match field observations. Similar competition avoidance behavior was found in *C. glomerata*, which avoids plants where its superior competitor, the specialist *C. rubecula* is

parasitizing *Pieris* host caterpillars (Geevliet, 1997). Such avoidance behavior may prevent aggregation of parasitoids, a phenomenon that is generally considered to play an important role in determining the stability or instability of parasitoid–host and predator–prey interactions (e.g., Hassell and May, 1974; Murdoch and Stewart-Oaten, 1989). Furthermore, active avoidance of competitors may aid in the coexistence of generalist and specialist species with overlapping niches.

Variation in the response to chemical cues during the process of host searching is another way in which responses to chemical cues can play a role in the coexistence of species. Such variation may result in the selection of different microhabitats or in different parts of the host population being attacked. Differences in responses to odor cues from microhabitats may even play a role in the reduction of gene flow between populations and thus in speciation (Vet and Janse, 1984).

Comparative studies with related species with comparable, preferably overlapping, niches are a strong tool for interpreting differences in responses of species to chemical cues. Parasitoids of *Drosophila* exploit the aggregation pheromones of adult flies (Wiskerke et al., 1993), possibly allowing them to track their host populations in space. A comparative study with a generalist and a specialist *Leptopilina* species showed differences in the specificity of the responses to pheromones of different *Drosophila* species (Hedlund et al., 1996). Again, such differences in responses to major foraging cues may play a role in niche differentiation between potential competing species, and hence in species coexistence. We are presently investigating the role of aggregation pheromones in the *Drosophila*-parasitoid interaction. Using an evolutionary approach, we are studying the possible ecological costs (increased mortality through natural enemies, increased competition after aggregation) and benefits (increased mate and food location) of aggregation pheromones for the flies and their exact role as kairomones for the exploitation of host populations by the parasitoids. We are especially interested in how the responses to pheromones play a role in determining the spatial heterogeneity of attack rates by parasitoids.

In conclusion, chemically mediated interactions can play a significant role at the population level. Since processes such as niche differentiation and competition between species can affect the structuring and functioning of communities, responses of individuals to chemical cues may have far-reaching consequences for processes at even higher levels of ecological organization.

THE TRITROPHIC APPROACH

A major development in parasitoid–host chemical ecology is related to the community level. For many years, predator–prey or parasitoid–host inter-

actions have been studied as bitrophic systems. In a seminal paper, Price et al. (1980) warned that natural enemy–herbivore and herbivore–plant interactions can only be fully understood in a multitrophic context in which plants, herbivores, and their natural enemies have evolved and function. Since then, the study of parasitoid–host interactions, and especially parasitoid foraging behavior, has expanded its focus towards tritrophic interactions. Including the plant level has opened an avenue of exciting research challenges. Developments started with the discovery that bean plants emit specific chemical volatiles when attacked by spider mites and that predatory mites are attracted to these volatiles (Dicke et al., 1990). Soon after, a similar situation was described for parasitoids (Turlings et al., 1990). For the sake of clarity I consider here plant–host-parasitoid systems, but generalizations to plant–prey–predator interactions can be made easily.

Plants can affect the impact of parasitoids on herbivorous hosts both indirectly (i.e., through effects on herbivore suitability) and directly, by providing parasitoids with food and shelter or by influencing their searching process. Especially at longer distances, plant stimuli guide the parasitoid in the process of host finding. Plants can provide the enemies of their enemies with chemical information about the presence and densities of herbivores. The benefit for parasitoids in responding to these plant cues is high, since the possibility to use host-derived information is limited because of strong selection on herbivores not to reveal themselves (Vet et al., 1991; Vet and Dicke, 1992).

A wealth of empirical data has been produced on this mode of indirect defense of plants. For reviews, see Vet and Dicke (1992), Tumlinson, et al. (1993), Turlings et al. (1993), Dicke (1994), and Dicke and Vet (1998). Chemical ecologists have set out to identify the plant chemical evolved, the biosynthetic pathways that underlie volatile productions, and the identification of the herbivore factors that initiate the induction process (e.g., Mattiacci et al., 1995; Alborn et al., 1997).

The fact that plants guide parasitoid host-searching has important evolutionary and ecological consequences for all trophic levels involved (see Dicke and Vet, 1998, for a more comprehensive discussion and specific references), as follows:

1. *For the Plant:* The fact that plants can actively recruit the enemies of their enemies links plant selection and foraging efficiency of parasitoids to plant fitness. This will influence the evolution of the plant defense mechanism (e.g., pay-off of investment in indirect versus direct defense).

2. *For the Herbivore:* Through plant selection, herbivores can influence their chance of being parasitized. Plant species, genotypes, even plant parts can vary in the production of volatiles that attract natural enemies. A less attractive plant (part) has a lower chance of being discovered or is searched less. This creates a partial refuge (enemy-free space), for the host, affecting the dynamics of the herbivore population and influencing the evolution of food-plant use by

herbivores. The fact that parasitoids can learn to focus on profitable plants (parts) will enhance these effects.

3. *For the Parasitoid:* Plants can affect host suitability (which determines important life history parameters of the parasitoid) and so direclty interfere with parasitoid dynamics. We can expect parasitoids to be adapted to the most prevailing and suitable plant–host complexes. Hence, parasitoid populations are expected to vary in their responses to important foraging cues, e.g., plant volatiles. Unfortunately there is still little knowledge of genetic variation in the use of plant cues by predators and parasitoids. In contrast, there is more insight into phenotypic plasticity in the use of infochemicals. This is not surprising since the variable nature of plant cues selects for flexibility in response patterns. After all, plant volatiles may vary due to abiotic factors. In addition, there is variation among plant species, plant genotypes, plant parts, and according to different biotic factors such as infestation by different herbivore species, stages and densities. This variation may hamper the evolution of genetically fixed responses in parasitoids, and we expect, and indeed find, much experience-related variability in the responses of parasitoids to plants.

AND ON TO FOOD WEBS

Even though the complexity of interactions under study has increased with the expansion from a bitrophic to a tritrophic focus, we may still be far from reality. Several ecologists propose that linear food chains, such as simple plant–herbivore–natural enemy systems, are an exception rather than a rule in nature. According to Polis and Strong (1996), we underestimate the breadth and diversity of phenomena found in natural systems by thinking in terms of discrete, homogeneous trophic levels. Food webs of most ecosystems are reticulate and interactions complex. Species may be omnivorous, feeding on species occupying more than one trophic level. Carnivore species may feed on one another (intraguild predation), thus killing potential competitors. Predators may change their diet as they grow. Generalist predators may affect interactions between prey species (apparent competition). Intraguild predation (IGP) has received increasing interest from ecologists because of its expected effect on community regulation. In the Hairston-Smith-Slobodkin model (Hairston et al., 1960), where distinct trophic levels are defined, predators regulate herbivores, allowing plant populations to grow. Or to put it simply: the more predators, the more plants. However, when predators eat other predators, it is more difficult to predict the effects of predators on prey populations (Polis and Holt, 1992). Experimental ecologists working in biological control have started to study the impact of IGP on the suppression of herbivore populations in the field (Rosenheim et al., 1993; Lucas et al., 1998).

Information conveyance through chemicals is expected to be an impor-

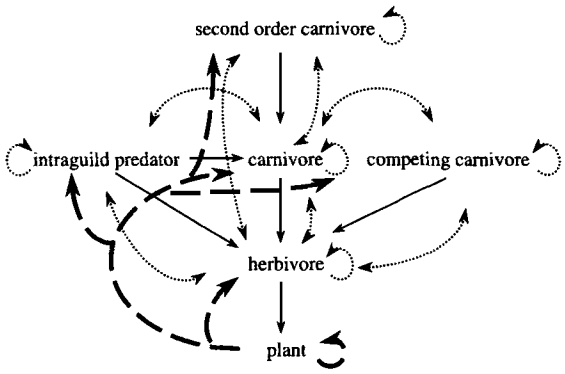

FIG. 2. The information web, based on infochemicals, is more complex than the food web. Solid straight lines indicate trophic interactions in the food web. Curved lines indicate the information web. Broken lines indicate information conveyance through herbivore-induced plant volatiles. Dotted lines indicate information conveyance through other infochemicals.

tant mediator of species interactions in food webs. Superimposed on a food web is an information web with even greater complexity since information conveyance occurs irrespective of trophic relationships (Figure 2). Natural selection is expected to shape the responses of the interacting species to chemical information on the presence and identity of important competitors and predators. Chemical ecologists have now started to unravel the chemically mediated interactions between plant-inhabiting arthropods as a basis to study the importance of indirect food web interactions in biological control systems (Pallini et al., 1997; Janssen et al., 1998).

EPILOGUE

The issues presented here are a personal, biased selection, mainly dealing with an integration of a mechanistic and functional approach to the use of infochemicals, how the use of infochemicals by individuals can affect population dynamics, and the possible role of infochemicals in structuring communities and species coexistence. I have not dealt with many other issues, such as the importance of increasing our knowledge of the genetic basis of infochemical use, or the exciting and promising use of molecular techniques in ecology. The latter has opened novel ways to explore genetic variation, competition, dispersion, relatedness between individuals and between species, costs and benefits of specific traits, etc. Much of this is likely to shape experimental ecology over the coming years. Where possible, molecular biologists, chemists, and experimental ecolo-

gists should collaborate to use these techniques for controlled ecological experimentation. In the field of plant–herbivore interactions, for example, the use of plants with reduced or enhanced expression of induced responses are successfully being used to answer questions on the functional significance of specific plant traits (Karban and Baldwin, 1997). Secondary plant metabolism, which plays a role in indirect defense of plants through the attraction of carnivores, may be more difficult to manipulate due to the complexity of the biosynthetic reactions. However, there is progress in manipulating plant traits by means of the plant's signal-transduction pathway (Karban and Baldwin, 1997), providing exciting experimental possibilities to study the costs of plant volatile production, measured in terms of plant fitness. In modern ecology, traditional separations between the different levels of ecological organization are fading rapidly (Figure 3). Ecological problems are increasingly approached in an integrative way, from the level of the gene to the ecosystem. This development is speeding up scientific progress and will strongly affect the field of chemical ecology. The marriage of chemistry with ecology is no longer a simple one. To tackle core problems in fundamental and applied ecology, there is an increasing need for interdisciplinary collaboration among chemists, ecologists, physiologists, molecular biologists, geneticists, and evolutionary biologists (Figure 3). An interdisciplinary approach is a requisite if we want to help preserve in nature what gives so much meaning to our studies—a great biodiversity of interacting organisms.

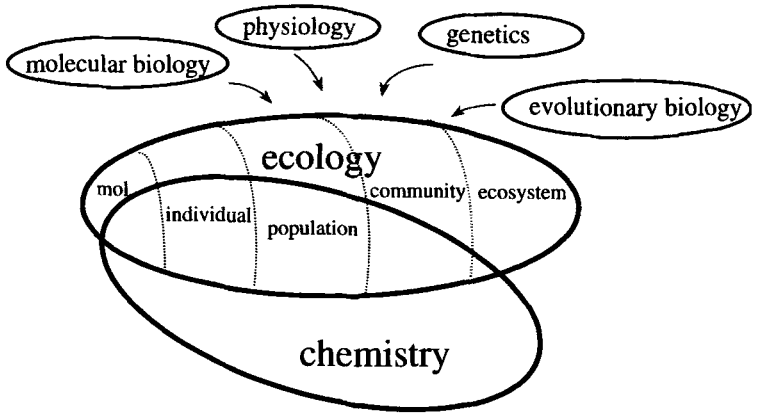

FIG. 3. Present developments and future: traditional separations between the different levels of ecological organization are fading. Chemical ecology is no longer limited to individual-level interactions but is including a greater variety of levels. Chemical ecology becomes more interdisciplinary in its approach.

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ELECTROPHYSIOLOGICALLY AND BEHAVIORALLY ACTIVE VOLATILES OF BUFFALO GOURD ROOT POWDER FOR CORN ROOTWORM BEETLES[1]

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Abstract—The dried, powdered roots of buffalo gourd, *Cucurbita foetidissima*, were tested in a cornfield and shown to attract adult northern and southern corn rootworm beetles. Coupled gas chromatography–electroantennography (GC-EAG) analyses of headspace samples of the root powder showed several GC-EAG-active compounds on the antennae of female northern, southern, and western corn rootworms. Among other techniques, solid-phase microextraction and GC-mass spectrometry identified the following GC-EAG-active compounds: hexanol, nonanal, 1-octen-3-ol, benzaldehyde, benzyl alcohol, (*E*)-3-octen-2-one, (*E,E*)-3,5-octadien-2-one, and (*E,Z*)-3,5-octadien-2-one. EAG dose–response studies of several of the identified root powder volatiles also were performed and compared with results from known attractants. Field tests of synthetic root powder volatiles in commercial cornfields showed that northern corn rootworm adults were attracted to (*E,E*)-3,5-octadien-2-one. The antennae of the *Diabrotica* species and the field tests showed specificity for different geometrical isomers of 3,5-octadien-2-one, with a behavioral preference for (*E,E*)-3,5-octadien-2-one. In addition, we have shown that the efficacy of buffalo gourd root powder as a feeding stimulant and arrestant can be enhanced for northern and western corn rootworm adults by augmenting buffalo gourd root powder with additional (*E,E*)-3,5-octadien-2-one.

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[1]The Editors apologize to Drs. Cossé and Baker. This paper should have appeared in the June 1998 issue (Volume 24, Number 6). By an error of filing, the paper was "lost" in the editorial office for some months.

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Key Words—Coleoptera, Chrysomelidae, buffalo gourd root powder, *Cucurbita foetidissima, Diabrotica virgifera virgifera, D. undecimpunctata howardi, D. barberi,* attractants, solid-phase microextraction, gas chromatography–electroantennography.

INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte; the southern corn rootworm, *D. undecimpunctata howardi* Barber; and the northern corn rootworm, *D. barberi* Smith and Lawrence (Coleoptera: Chrysomelidae) are among the more economically important damaging insect pests of corn, *Zea mays* L., in the United States (Metcalf, 1986). It is estimated that control costs and crop losses due to *Diabrotica* spp. approach $1 billion annually (Metcalf, 1986). Applications of soil insecticides to reduce the larval densities in the root zone are the primary management practice for *Diabrotica* spp. in continuous corn (Mayo and Peters, 1978; Mayo, 1986). Baits containing a combination of cucurbitacins, volatile attractants, and small quantities of insecticide have shown promise as an alternative management tool that targets adult beetles (Metcalf et al., 1987; Lance and Sutter, 1990, 1991, 1992; Weissling and Meinke, 1991a,b). The nonvolatile, oxygenated, tetracyclic triterpenoid cucurbitacins found in many species of Cucurbitaceae evoke compulsive feeding and locomotory arrestment in diabroticite beetles (Metcalf et al., 1980, 1982). Adding a volatile attractant, such as a mixture of 1,2,4-trimethoxybenzene, indole and (*E*)-cinnamaldehyde (compounds emitted by the flowers of Cucurbitaceae) to a formulation of the powdered roots of the buffalo gourd, *Cucurbita foetidissima* H. B. K., enhances the efficacy of the bait (Metcalf et al., 1987; Lance, 1988a,b; Weissling and Meinke, 1991a,b).

This study presents the results of experiments designed to determine whether the dried, powdered roots of the buffalo gourd contain volatiles that are electrophysiologically active and to see whether these naturally occurring compounds can attract *Diabrotica* beetles, thereby potentially increasing the effectiveness of the current toxicant-feeding baits for *Diabrotica* species.

METHODS AND MATERIALS

Insects. Male and female western and southern corn rootworm adults were obtained from a rearing facility operated by French Agricultural Research, Inc., Lamberton, Minnesota. In addition, western, southern, and northern corn rootworm adults were collected from cornfields in and around the Iowa State University campus. Male and female beetles were placed in separate cages and kept at 25°C under a 14L:10D photoperiod. Adults had access to water and slices of squash.

Collection of Buffalo Gourd Root Powder Volatiles. Buffalo gourd root powder was obtained from MicroFlo Company (Lakeland, Florida). Headspace collections of volatiles from the powder were obtained by three different methods. In the first method, which does not require the use of solvents, buffalo gourd root powder volatiles were collected directly from 2-dram (7.4-ml) sample vials by using solid-phase microextraction (SPME) (Supelco, Inc., Bellefonte, Pennsylvania). The SPME unit consisted of a fiber coated with 100 μm of polydimethylsiloxane housed within a syringe. The fiber was extruded from the syringe and inserted into the headspace of a 2-dram vial containing 1.5 g of buffalo gourd root powder at room temperature (ca. 25–28°C). After 20 min it was retracted and immediately inserted into the injector port (250°C) of a HP 5890 (Hewlett-Packard Comp., Palo Alto, California) gas chromatograph (GC), and thermally desorbed.

The second method (no solvents needed), involved placing 1 g of buffalo gourd root powder in a 25-ml fritted sparger that was connected to a Tekmar 2000 purge and trap concentrator fitted with a turbocool unit (Tekmar, Cincinnati, Ohio). The conditions for collecting volatiles on a cooled (−20°C) Tenax trap, housed in the Tekmar 2000 instrument, were as follows: 2 min prepurge, sample at 50°C, 2 min preheat, 10 min purge (He, 20 ml/min), preheat desorb at 195°C, and 4 min desorb at 200°C. The Tekmar 2000 was directly interfaced to the coupled gas chromatograph–electroantennograph (GC-EAG) setup (see below).

For the third method, 35 g buffalo gourd root powder samples were placed in a two-necked 250-ml flask. Charcoal-filtered air was blown over the powder at a rate of 10 ml/sec for 9 hr at room temperature. Volatiles were collected in a glass tube (10 cm long × 4 mm ID) containing 350 mg of precleaned Tenax TA (Alltech Associates, Inc., Deerfield, Illinois) placed between two glass wool plugs. The Tenax TA trap was desorbed with 3 ml of redistilled HPLC-grade hexane. Samples were stored at −20°C for later analyses by GC-EAG and coupled GC-mass spectrometry (GC-MS).

Chemical Analyses. Collected volatiles resulting from all three methods were injected in splitless mode on a 30-m × 0.25-mm-ID fused silica capillary gas chromatographic column (DB-1, DB-5, or DB-225 model, J&W Scientific, Folsom, California) for analysis by GC-EAG and GC-MS. Column conditions were as follows: He carrier gas flow of 1.5 ml/min, injector temperature 250°C, 1 min delay on inlet purge, and 4 min at 35° C then 25°C/min to 320°C (DB-1) or 250°C (DB-5) or 10°C/min to 220°C (DB-225). The SPME samples were analyzed by using the above-mentioned conditions with a 4-min delay on inlet purge. GC-MS analyses were performed by using the HP 5890 gas GC with a direct interface to an Hewlett-Packard 5972 mass selective detector (electron impact, 70 eV).

Electroantennogram Responses. Coupled gas chromatography–electroantennography and EAG analyses were performed according to standard methods (e.g., Cossé et al., 1995; Cossé and Baker, 1996) by using an HP 5890 GC. EAG recordings were made by inserting a glass pipet Ag–AgCl saline electrode

(World Precision Instruments, Sarasota, Florida) into the back of an excised beetle head. A second saline recording electrode was placed in contact with the distal end of one antenna. Both pipets were filled with Beadle-Ephrussi saline (Ephrussi and Beadle, 1936). To examine antennal sensitivity to the identified buffalo gourd root powder volatiles, we recorded EAGs that were not coupled to the GC from female western and southern corn rootworm beetle antennae in response to a dose–response series of commercial compounds. In addition, EAGs were obtained from male and female antennae of northern and western corn rootworm beetles to a 100-μg dose of selected synthetic compounds. Serial dilutions of the tested compounds were made in HPLC-grade methylene chloride such that the tested compounds were applied to filter paper strips (0.5 cm $\times$ 3.0 cm, Whatman No. 1) in 10 μl of solvent. The filter paper strips were placed inside glass Pasteur pipets (15 cm long). The antenna was continuously flushed with a charcoal-filtered, humidified airstream. The air, flowing at a rate of 20 ml/sec, was delivered through a glass tube (8 mm ID) ending 10 mm in front of the preparation. Two milliliters of volatiles from the stimulus pipet were puffed into the constant airstream by a mechanical puffing device (Syntech, Hilversum, The Netherlands), delivering puffs of 0.1-sec duration. The test compound was injected into the airstream 15 cm upstream from the antenna. Solvent blank puffs (filter paper plus solvent) were presented to each EAG preparation before and after each dose level of test compound. The EAG amplitudes in response to the synthetic compounds were corrected for mechanoreceptive response by subtracting the mean amplitudes of the EAGs generated from the solvent blank puffs. Within a particular series of test compounds, presentation order of the test compounds was randomized within doses, beginning with the 0.01-μg odor cartridges and working upward to the 10-μg odor cartridges. The resulting EAG data were subjected to analysis of variance (ANOVA) and mean responses were compared by the LSD method (Sokal and Rohlf 1981).

Chemicals. Indole, benzyl alcohol, benzaldehyde, and hexanol were obtained from Sigma Chemical Co. (St. Louis, Missouri). The compounds 1-octen-3-ol and nonanal were purchased from Bedoukian (Danbury, Connecticut) and (*E*)-cinnamaldehyde and 1,2,4-trimethoxybenzene were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Samples of (*E*)-3-octen-2-one and (*E*,*E*)-3,5-octadien-2-one, in quantities sufficient for physiological and behavioral tests, were synthesized by W. L. Roelofs, Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York. The (*E*,*E*)-3,5-octadien-2-one came in several batches, containing different proportions of the 3,5-octadien-2-one isomers (see Figure 1 for additional details). Initial samples of authentic 3,5-octadien-2-one for identification were kindly supplied as a gift by Joerg D. Hardege (Gatty Marine Laboratory, St. Andrews, Fife, Scotland).

Field Tests. In August 1994, prior to the chemical and physiological anal-

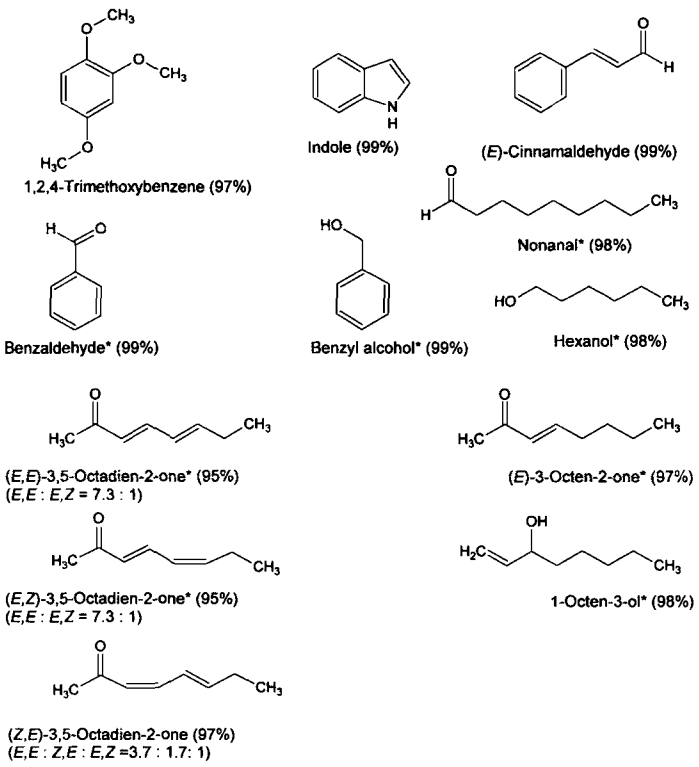

FIG. 1. Structure and purity (in parentheses) of test compounds. Asterisks indicate compounds identified in the headspace of buffalo gourd root powder.

yses, buffalo gourd root powder was tested for attracting corn rootworm beetles on a small experimental corn plot (40 × 60 m) located on the Iowa State University campus. Five grams of compacted buffalo gourd root powder were placed in sachets made of several layers of cheesecloth and affixed to the glue of yellow Pherocon A. M. sticky traps (Trécé, Inc., Salinas, California). A control trap received no treatment. Traps were affixed to corn plants at about ear height, 20 m apart, around the edges of the experimental plot. Five replicate pairs were left in the field for 24 hr, after which time they were replaced by new traps and fresh baits. The 24-hr trapping period was repeated five times (August 5–9) with corn at late milk to early dough stage. Trap catch data was transformed [log $(x + 1)$] to normalize variances and subjected to two-way ANOVA with replication.

In August 1996, two different sets of field tests were performed. In the first test, several identified EAG-active compounds were tested in a commercial cornfield located in Story County, Iowa. Traps were baited with candidate attrac-

tants (dissolved in acetone) that were applied to cotton dental rolls (3.8 cm long × 1 cm diam., Peerless Int., Inc., Massachusetts) as a 0.8-ml mixture of acetone and mineral oil (1 : 1). Control traps were baited with dental rolls that were only treated with the acetone–mineral oil mixture. Trap catches in response to EAG-active compounds were compared with similarly prepared known attractant mixtures containing 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde (1 : 1 : 1). Traps were deployed at ear height, 30 m apart, in a randomized complete block design with six replicates that were placed along the edges of the cornfield. Traps were left in the field for 72 hr during August 20–22 with corn at late milk to early dough stage.

In the second test, we compared the numbers of corn rootworm beetles arriving on corn leaves that were sprinkled with buffalo gourd root powder and buffalo gourd root powder augmented with a wick containing synthetic volatile attractant. A salt shaker was used to sprinkle four corn leaves, two below and two above the corn ear with 200 mg of buffalo gourd root powder. Dental rolls containing 30 mg of 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde (1 : 1 : 1) applied as a 0.5-ml mixture of acetone and mineral oil (1 : 1) were affixed to some of these corn plants at ear height. Other plants receive dental rolls that were only treated with the acetone–mineral oil mixture. Treated plants were laid out in a randomized complete block design with five replicates with blocks and plants 15 m apart, starting on the fourth row from the edge of the field. Beetles were counted on the four treated and the four control leaves 5 hr after the start of the experiment. Corn was at milk stage during this test (August 21). The same experiment was repeated a year later with the exception that the synthetic attractant was replaced by 100 mg of (*E*,*E*)-3,5-octadien-2-one (*E*,*E*:*E*,*Z* = 7.3 : 1). Corn was at silking stage during this test (July 28, 1997). Data were transformed [log $(x + 1)$] to normalize variances and subjected to ANOVA, means were compared using the Student-Newman-Keuls test. Untransformed data are presented in the tables.

RESULTS

Field Trapping with Buffalo Gourd Root Powder. Analysis of variance of the mean numbers of beetles that were trapped per day in the small-plot field test indicated significantly higher numbers of southern corn rootworm beetles ($F = 35.30$; $df = 4, 40$; $P \ll 0.001$) and northern corn rootworm beetles ($F = 8.59$; $df = 4, 40$; $P \ll 0.001$) in buffalo gourd root powder-baited traps than in control traps. The daily averages (±SD) were the highest for southern corn rootworm beetles (7.12 ± 4.84) followed by northern corn rootworm beetles (1.92 ± 1.68), and western corn rootworm beetles (1.48 ± 0.87), respectively. The daily control

averages (±SD) for southern, northern, and western corn rootworm beetles were 0.76 ± 0.88, 0.92 ± 0.91, and 0.28 ± 0.29, respectively. Because the beetles did not contact the root powder itself, these results indicated that behaviorally active volatiles were being emitted from the buffalo gourd root powder.

Analyses of Buffalo Gourd Root Powder Volatiles. The GC profiles obtained by the three methods used to collect buffalo gourd root powder volatiles did not reveal any prominent qualitative variation. However, each method had its own advantages. With the SPME technique, headspace samples could be analyzed quickly (20 min) by both GC-MS and GC-EAG without the presence of solvent. Interfacing the purge and trap method with the GC-EAG allowed for higher amounts of trapped volatiles to be transferred to the GC column and subsequently to the antenna of the beetle. However, sample analysis by this method was limited to the GC-EAG setup and took a relatively long time (1 hr). The adsorption of buffalo gourd root powder volatiles on Tenax TA allowed for relatively large quantities to be collected and stored for later analysis by both GC-EAG and GC-MS. However, solvent used to desorb collected volatiles could possibly interfere with GC peaks of interest, and this setup also required testing the trap for breakthrough and precleaning the adsorbent.

Thirty-six GC-EAG analyses of volatiles, equally distributed over the three collection methods and the three GC columns, collected from buffalo gourd root powder were obtained from 18 female southern corn rootworm and 18 female western corn rootworm antennae. Only those GC peaks that consistently elicited simultaneous EAG activity were targeted for further analysis. These GC-EAG analyses consistently revealed 14 GC peaks on all three GC columns having corresponding EAG activity on both female southern corn rootworm and western corn rootworm antennae (Figure 2; Table 1). The retention times and GC-MS spectra of eight synthetic compounds presented in Table 1 were identical to those of peaks 2, 5, 8, 9, 10, 12, 13, and 14 detected in the headspace of buffalo gourd root powder on all three columns. Results from an additional four GC-EAG analyses from four different female northern corn rootworm antennae by using the SPME collection method on DB-1 and DB-225 columns showed GC-EAG activity identical to that obtained with southern corn rootworm and western corn rootworm antennae. Using three different isomers of 3,5-octadien-2-one, GC-EAG and GC-MS analyses revealed that only the *E,E* and *E,Z* isomers, and not the *Z,E* isomer, evoked EAG responses regardless of which of the three corn rootworm species' antennae were used.

Electroantennogram Responses. The EAG responses of female western and southern corn rootworm antennae to puffs of three GC-EAG-active compounds plus three known adult corn rootworm attractants were recorded at four dosages (Figure 3). The EAG amplitudes increased as the dosages in the cartridges

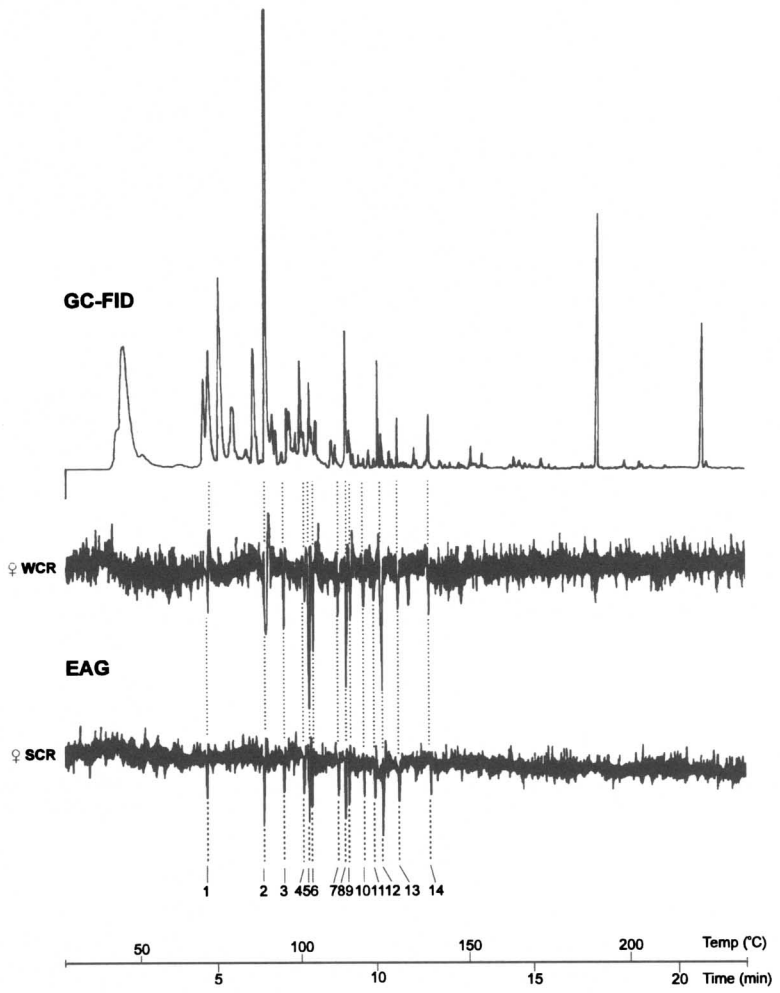

FIG. 2. Simultaneously recorded gas chromatogram [flame ionization detector (FID)] of buffalo gourd root powder volatiles collected by solid-phase microextraction and electroantennograms (EAG) from female western (WCR) and southern corn rootworm (SCR). Numbered peaks indicate EAG-active compounds (see also Table 1).

increased from 0.01 to 10 μg for all compounds tested, except when female southern corn rootworm antennae were exposed to 1,2,4-trimethoxybenzene. Statistical analyses of the dose–response data showed that both female western and southern corn rootworm antennae gave significantly higher responses to benzyl alcohol and indole than to the other four compounds tested.

TABLE 1. IDENTIFIED BUFFALO GOURD ROOT POWDER VOLATILES WITH ELECTROPHYSIOLOGICAL ACTIVITY ON FEMALE WESTERN, SOUTHERN, AND NORTHERN CORN ROOTWORM ANTENNAE

Peak no.[a]	Compound	Identification[b]
1	unknown	—
2	hexanol	GC-MS
3	cyclohexanol	MS
4	cyclooctanone	MS
5	1-octen-3-ol	GC-MS
6	aromatic hydrocarbon	MS
7	aromatic hydrocarbon	MS
8	benzaldehyde	GC-MS
9	(*E*)-3-octen-2-one	GC-MS
10	nonanal	GC-MS
11	aromatic hydrocarbon	MS
12	(*E*,*Z*)-3,5-octadien-2-one	GC-MS
13	(*E*,*E*)-3,5-octadien-2-one	GC-MS
14	benzyl alcohol	GC-MS

[a]Numbers correspond to labeled peaks in Figure 2.
[b]Identification is based on a mass spectrum library (Wiley 138K) search (MS) and/or comparison of mass spectra and retention times of the natural materials with those of the identified synthetic compounds (GC-MS).

Comparison of EAG responses between female western and southern corn rootworm antennae indicated significantly higher responses of female western corn rootworm antennae to benzaldehyde ($F = 48.8$; $df = 1, 32$; $P \ll 0.001$) benzyl alcohol ($F = 22.2$; $df = 1, 32$; $P \ll 0.001$), and indole ($F = 27.6$; $df = 1, 32$; $P \ll 0.001$), than were obtained from southern corn rootworm antennae. Significantly higher EAG responses were evoked in response to (*E*)-cinnamaldehyde from southern corn rootworm antennae ($F = 121.43$; $df = 1, 32$; $P \ll 0.001$) than from western corn rootworm antennae.

Comparisons of the EAG responses of male and female antennae of northern and western corn rootworms to single dosages of 1-octen-3-ol, (*E*,*E*)-3,5-octadien-2-one, and indole showed no significant sex differences, and male and female responses were therefore pooled for further analysis (Figure 4). The EAG responses of northern corn rootworm antennae were significantly higher to 1-octen-3-ol and (*E*,*E*)-3,5-octadien-2-one than to indole ($F = 54.8$; $df = 2, 57$; $P \ll 0.001$). Similar results were obtained with western corn rootworm antennae ($F = 30.4$; $df = 2, 57$; $P \ll 0.001$). Comparison between the EAG responses of northern and western corn rootworm antennae to 1-octen-3-ol, (*E*,*E*)-3,5-octadien-2-one, and indole revealed no significant differences ($F = 1.3$; $df = 1, 114$; $P = 0.26$).

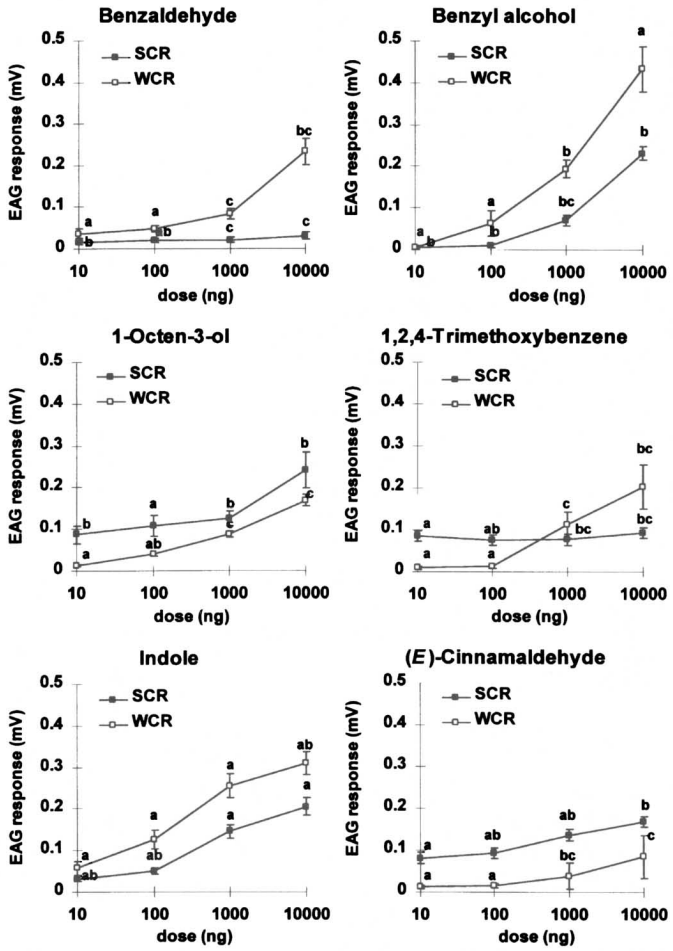

FIG. 3. Electroantennogram (EAG) dose–response of female western (WCR) and southern corn rootworm (SCR) antennae to selected volatile compounds emitted from buffalo gourd root powder. Stimuli were synthetic standards. Standard deviations of mean responses are presented as error bars (N = 5). The EAG responses of WCR and SCR antennae for each dosage were compared by ANOVA, and if significant at $P < 0.05$, the differences among means were tested for significance according to the LSD method.

Field Trapping. The mean number of corn rootworm beetles captured on traps baited with selected GC-EAG active buffalo gourd root powder volatiles is presented in Table 2. As expected, a mixture of 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde (1 : 1 : 1) attracted significantly more north-

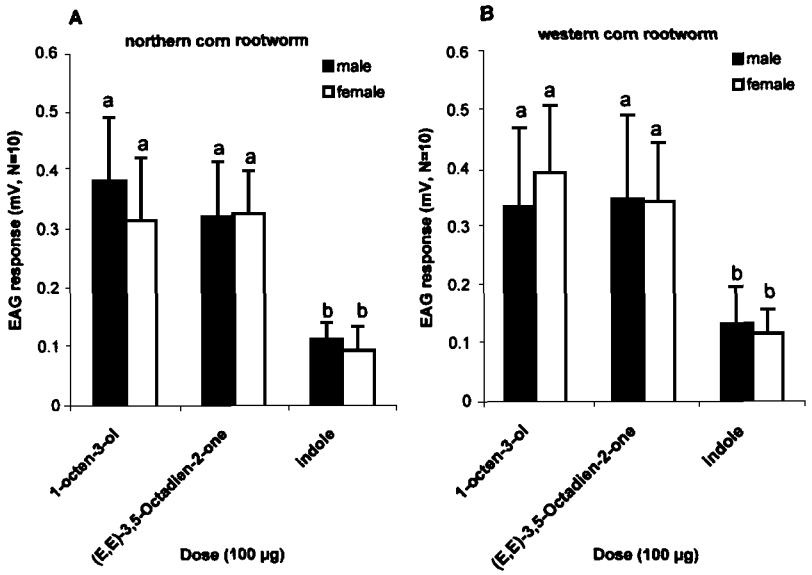

FIG. 4. Electroantennogram (EAG) responses of male and female western (WCR) and northern corn rootworm (NCR) antennae to a single dose (100 μg) of selected volatile compounds emitted from buffalo gourd root powder. Stimuli were synthetic standards. Standard deviations of mean responses are presented as error bars. The EAG responses were compared by ANOVA, and if significant at $P < 0.05$, the differences among means were tested for significance according to the LSD method.

ern, western, and southern corn rootworm beetles than did an unbaited control. When beetle captures on traps baited with isomeric mixtures of 3,5-octadien-2-one as well as with a 1 : 1 mixture of (*E*)-3-octen-2-one and 3,5-octadien-2-one were compared with captures on control traps, only one isomeric mixture of 3,5-octadien-2-one attracted significantly more beetles than did the control and attraction was limited to northern corn rootworms. Although there was no significant difference in the number of northern corn rootworms attracted to the two isomer mixtures of 3,5-octadien-2-one, only the 7.3 : 1 (*E*,*E*:*E*,*Z*) mixture attracted significantly more beetles than did the control. Traps baited with (*E*)-3-octen-2-one or a 1 : 1 mixture of (*E*)-3-octen-2-one and 3,5-octadien-2-one (3.7 : 1, *E*,*E*:*E*,*Z*) captured northern corn rootworm beetles, but the numbers were not significantly different from those of the control traps. No western or southern corn rootworms were captured on the yellow-sticky control traps, whereas, on average, 3 (±1.7 SD) northern corn rootworms were captured on the controls. The number per plant (±SD) of northern, western, and southern corn rootworm beetles on four leaves of 10 corn plants

TABLE 2. MEAN NUMBERS OF NORTHERN (NCR), WESTERN (WCR), AND SOUTHERN CORN ROOTWORM (SCR) ADULTS CAPTURED ON YELLOW PHEROCON A. M. STICKY TRAPS BAITED WITH CHEMICALS DERIVED FROM BUFFALO GOURD ROOT POWDER, STORY COUNTY, IOWA, AUGUST 20–22, 1996[a]

Treatment (100 mg/compound)[b]	Capture/trap (mean ± SD)		
	NCR	WCR	SCR
1,2,4,-Trimethoxybenzene + indole + (*E*)-cinnamaldehyde (1 : 1 : 1)	70.2 ± 30.7a	5.5 ± 4.5a	2.3 ± 1.0a
(*E*,*E*)-3,5-Octadien-2-one (3*E*,5*E* : 3*E*,5*Z* = 7.3 : 1)	11.8 ± 3.3b	0.2 ± 0.4b	0.2 ± 0.4b
(*E*,*E*)-3,5-Octadien-2-one (3*E*,5*E* : 3*E*,5*Z* = 3.7 : 1)	8.0 ± 5.6bc	0.2 ± 0.4b	0.2 ± 0.4b
(*E*)-3-Octen-2-one	5.8 ± 3.8c	0.0 ± 0.0b	0.0 ± 0.0b
(*E*)-3-Octen-2-one + (*E*,*E*)-3,5-Octadien-2-one (3*E*,5*E* : 3*E*,5*Z* = 3.7 : 1)	5.8 ± 2.6c	0.0 ± 0.0b	0.0 ± 0.0b
Control	3.0 ± 1.7c	0.0 ± 0.0b	0.0 ± 0.0b
F statistics	25.3[c]	24.9[c]	26.0[c]

[a]Corn stage; late milk-early dough (R3-R4). Means within columns followed by the same letter do not differ by Student-Newman-Keuls test, $P > 0.05$.
[b]Compounds were applied on dental wicks as an acetone-mineral oil mixture (0.8 ml, 1 : 1).
[c]Statistical significance at $P < 0.001$ ($df = 5, 25$).

just prior to the start of the experiment was 0.8 (±0.9 SD), 0.3 (±0.8 SD), and 0.1 (±0.6 SD), respectively. An overall lower capture of western and southern compared with northern corn rootworm (Table 2) might be due to the somewhat higher population density of northern corn rootworm indicated in the beetle-per-plant counts.

The mean numbers of corn rootworm beetles observed on corn leaves treated with buffalo gourd root powder with or without the presence of a wick containing synthetic attractant are presented in Table 3. As expected, leaves treated with buffalo gourd root powder had significantly more western and northern corn rootworm beetles than did the untreated control leaves. The addition of a point-source wick of the synthetic attractants significantly increased the numbers of these beetles per corn plant compared with those found on plants only treated with the buffalo gourd root powder. With either the mixture of 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde or the newly identified (*E*,*E*)-3,5-octadien-2-one, the mean number of western corn rootworms per four leaves increased by a factor of 2.8. Similar significant trends were observed for the northern corn rootworm. However, overall beetle counts were lower than those of the western corn rootworm. For the southern corn rootworms, no significant differences were observed.

TABLE 3. MEAN NUMBERS OF WESTERN (WCR), NORTHERN (NCR), AND SOUTHERN CORN ROOTWORM (SCR) ADULTS COUNTED ON CORN LEAVES AFTER APPLICATION OF BUFFALO GOURD ROOT POWDER (BGRP) EITHER WITH OR WITHOUT DIFFERENT CHEMICAL ATTRACTANTS APPLIED TO A COTTON WICK AFFIXED TO THE PLANT

Treatment[b]	Counts/plant (mean ± SD)[a]		
	WCR	NCR	SCR
August 21, 1996			
BGRP + TIC	30.0 ± 16.6a	2.0 ± 1.2a	0.2 ± 1.2a
BGRP	7.6 ± 3.4b	1.4 ± 1.1ab	0.0 ± 0.0a
Control	1.6 ± 1.9c	0.0 ± 0.0b	0.0 ± 0.0a
F statistics[c]	19.5**	15.2*	NS
July 28, 1997			
BGRP + (*E*,*E*)-3,5-octadien-2-one	21.4± 11.5a	1.4 ± 1.1a	0.4 ± 0.5a
BGRP	7.6 ± 4.2b	0.4 ± 0.5b	0.4 ± 0.5a
Control	3.0± 1.9c	0.0 ± 0.0b	0.2 ± 0.4a
F statistics[c]	17.2**	5.2*	NS

[a]Beetle counts were made on four corn leaves, two above and two below the corn ears, 5 hr after application. Corn stage August 21, 1996: milk (R3); corn stage July 28, 1997: early silking (R1).
[b]BGRP = 200 mg applied over four leaves; TIC = 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde, 30 mg (1 : 1 : 1); (*E*,*E*)-3,5-octadien-2 one = 100 mg (3*E*,5*E* : 3*E*,5*Z* = 7.3 : 1). Chemicals were applied on dental wicks as acetone-mineral oil mixtures (0.5 ml, 1 : 1) and affixed to the corn stalk at ear height.
[c]Asterisks denote statistical significance at $P < 0.05$ (*) and $P < 0.001$ (**), NS denotes $P > 0.05$ ($df = 2, 8$). Means within dates and columns followed by the same letter do not differ by Student-Newman-Keuls test, $P > 0.05$.

DISCUSSION

Our results demonstrate that volatiles from the dry, powdered roots of the buffalo gourd can attract corn rootworm adults in the absence of an additional synthetic attractant. Furthermore, our approach of combined GC-EAG analyses demonstrated that several volatile constituents of the buffalo gourd root powder are EAG-active on the antennae of western, northern, and southern corn rootworm beetles and that at least one of these volatiles, (*E*,*E*)-3,5-octadien-2-one, is capable of attracting northern corn rootworms in significant numbers in field trapping experiments. In addition, we have shown that the arrival and retention of northern and western corn rootworm beetles on leaves sprinkled with buffalo gourd root powder can be enhanced with additional (*E*,*E*)-3,5-octadien-2-one presented on a cotton wick.

Adult corn rootworms are attracted to volatile compounds from corn plants and to volatiles isolated or derived from the blossoms of *Cucurbita* species (Hammack, 1996, 1997; Prystupa et al., 1988, Metcalf et al., 1995; Metcalf and

Metcalf, 1992, and references therein), and several studies have shown that contact of the beetles with the nonvolatile cucurbitacins present in buffalo gourd root powder can be increased with the addition of synthetic attractants identified from flowers (Metcalf et al., 1987; Lance and Sutter, 1990, 1991, 1992; Weissling and Meinke, 1991a,b).

Several of the volatile EAG-active compounds identified in our headspace samples of the buffalo gourd root powder previously have been identified from *Cucurbita* flowers and tested as attractants for *Diabrotica* species in the field. For example, benzaldehyde and benzyl alcohol, identified in this study from root powder, are attractive to several *Diabrotica* species, but only at relatively high dosages (>200 mg) (Lampman et al., 1987; Metcalf and Lampman, 1989; Metcalf and Metcalf, 1992). A compound previously identified from corn, 1-octen-3-ol, also identified from root powder in this study, was not attractive to northern or western corn rootworms (Hammack, 1997). However, the identified EAG-active volatiles, (*E,E*)-3,5-octadien-2-one, (*E,Z*)-3,5-octadien-2-one, and (*E*)-octen-2-one, had not been previously identified or tested for attractancy. Our EAG results showed specificity for all three beetle species for two of the three tested geometrical isomers of 3,5-octadien-2-one. Significant EAG activity occurred only in resposne to the *E,E* and *E,Z* isomers, and not to the *Z,E* isomers. This specificity also was expressed behaviorally where mixtures with different ratios of *E,E* and *E,Z* isomers attracted northern corn rootworms in the field, but only traps baited with mixtures higher in *E,E* content attracted significantly more beetles than the control traps. (*E*)-Octen-2-one did not attract significant numbers of corn rootworm beetles, nor were any beetles attracted when (*E*)-octen-2-one was tested in combination with one of the isomer mixtures of 3,5-octadien-2-one.

That buffalo gourd root powder alone is capable of attracting *Diabrotica* species could be important information when synthetic attractants in combination with formulations of buffalo gourd root powder are evaluated as baits for controlling adult corn rootworms. Without the proper controls, the presence of buffalo gourd root powder could unduly increase the effectiveness of the tested attractant.

Corn silk has been shown to attract western and northern corn rootworms in wind-tunnel tests (Prystupa et al., 1988), and several studies on field attractancy of *Diabrotica* species to volatile compounds have noted significantly decreased trap catch during the silking stage of the corn (Hammack, 1996; Lampman et al., 1987; Andersen and Metcalf, 1986). This decrease in trap catch could be due to the presence of (*E,E*)-3,5-octadien-2-one. This compound was recently identified together with two other volatile compounds from senescing silk that protrudes from the tips of the corn ears and was shown to be EAG-active on western corn rootworm antennae (Hibbard et al., 1997a). Our beetle counts on corn leaves, dusted with buffalo gourd root powder and augmented with the

presence of additional (*E,E*)-3,5-octadien-2-one, were obtained during the silking period of the corn. However, we observed beetles feeding on the silks as well as on the buffalo gourd root powder particles of corn plants that received the buffalo gourd root powder–attractant combination.

After submission of this paper it came to our attention that (*E,E*)-3,5-octadien-2-one had been identified from extracts of buffalo gourd root powder and was shown to be EAG-active on western corn rootworm antennae (Hibbard et al., 1997b). These results corroborate our EAG findings. However, additional research is needed to study the novelty of (*E,E*)-3,5-octadien-2-one as an attractant for corn rootworm beetles as well as the effects of blends of all identified attractants in the headspace of buffalo gourd root powder. Studies to examine whether these compounds alone or in combination can increase the feeding stimulant effect of buffalo gourd root powder in commercial cornfields at different crop stages could contribute to the efficacy of the toxicant-feeding stimulant formulations for controlling adult *Diabrotica* beetles.

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PATTERNS OF OVIPOSITION STIMULANTS FOR CARROT FLY IN LEAVES OF VARIOUS HOST PLANTS

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Abstract—Undamaged leaves of 12 host-plant species differing widely in acceptability to ovipositing carrot flies were extracted with a microwave-assisted method with hexane as solvent. The highly stimulatory diethyl ether fraction obtained by separation on a silica gel column was semiquantitatively analyzed by GC-MS for previously identified oviposition stimulants of the carrot fly (phenylpropenes, fluranocoumarins, polyacetylenes). Various plant species exhibited widely differing profiles of these compounds. In choice assays, moderate numbers of eggs were deposited underneath surrogate leaves sprayed with fractions that contained high amounts of just one type of compound and low amounts of the other two types. Only fractions with medium to high levels of at least two compound classes elicited strong ovipositional responses (e.g., phenylpropenes and polyacetylenes in *Daucus carota*, furanocoumarins and polyacetylenes in *Heracleum sphondylium* and *Conium maculatum*). None of the examined plants contained high quantities of all three compound classes. The contents of the stimulants seemed to account in a synergistic manner for the variation in activity of the diethyl ether fraction. However, they could not explain adequately the observed preference hierarchy of the carrot fly for the host-plant species.

Key Words—Phenylpropenes, furanocoumarins, polyacetylenes, coumarins, Apiaceae, *trans*-methylisoeugenol, *trans*-asarone, xanthotoxin, bergapten, falcarinol, falcarindiol, *Psila rosae*, Diptera, Psilidae.

INTRODUCTION

The carrot fly, *Psila rosae* (F.) (Diptera, Psilidae), is an oligophagous insect; more than a 100 plant species, exclusively belonging to the family Umbel-

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liferae (Apiaceae), have been recorded as hosts (Hardman et al., 1990; Ellis et al., 1992). The female flies perform an exploratory run over the leaves before accepting a host plant and depositing their eggs in the soil underneath. The larvae feed on the roots and may cause severe damage to various crops (carrots, celery root, parsnip). Consistent differences in resistance among carrot cultivars have been established (Ellis and Hardman, 1981; Ellis et al., 1984), but relatively few studies have dealt with the underlying mechanisms. Evidence has been put forward for the involvement of both antibiosis (Guerin et al., 1981; Maki and Ryan, 1989) and antixenosis (Guerin and Ryan, 1984; Guerin and Städler, 1984).

Host selection in the carrot fly is influenced by physical (leaf shape and color) and chemical plant cues (Städler, 1977; Degen and Städler, 1996). Semiochemicals in the surface wax of carrot leaves were shown to stimulate oviposition (Städler and Buser, 1984). The compounds identified belong to three classes (phenylpropenes, furanocoumarins and substituted coumarins, polyacetylenes) that are in combination characteristic of the umbellifers (Städler, 1986). Previous studies have failed to correlate the varying level of antixenotic resistance among carrot cultivars with the amounts of the known oviposition stimulants on the leaf surface (Visser and de Ponti, 1983; Guerin and Städler, 1984; Städler et al., 1990). In addition, celery root, another important host, exhibited a pattern of stimulatory compounds in its leaf surface distinct from that of carrot (Städler et al., 1990). This prompted us to study various other host-plant species that differ more markedly in acceptability than carrot cultivars (Degen and Städler, unpublished). In the present study, leaf surface extracts of 12 host-plant species were semiquantitatively analyzed by high-resolution gas chromatography–mass spectrometry (HRGC-MS) for six previously identified oviposition stimulants. The acceptabilities of real leaves and the relative stimulatory activities of the fractions examined were compared to the respective amounts of stimulants detected in order to assess the importance of these compounds for host acceptance of the carrot fly.

METHODS AND MATERIALS

Plants. Twelve host-plant species were chosen for examination (Table 1), including the two previously studied carrot cultivars, Danvers and Sytan (Guerin and Städler, 1984; Städler et al., 1990). Foliage was collected from locally occurring wild plants (*Heracleum sphondylium*, *Aegopodium podagraria*, *Athyrium filix-femina*) and from plants grown in pots (*Pimpinella major*) or in seed beds (the eight remaining species) at the Swiss Federal Research Station, Wädenswil. The young plants were heavily damaged during a hailstorm, but recovered and looked healthy by the time they were used for oviposition assays and extractions, i.e., about one month (plant set 2) and two months (plant set 1) later. Only the annual plants *Anethum graveolens* and *Anthriscus cerefolium* had to be resown.

TABLE 1. SEMIQUANTITATIVE GC-MS ANALYSIS OF FRACTION 3.1 FOR SIX PREVIOUSLY IDENTIFIED OVIPOSITION STIMULANTS[a]

Set	Plant species	Phenylpropenes		Furanocoumarins		Polyacetylene	
		t-Methyl-isoeugenol	*t*-Asarone	Xanthotoxin	Bergapten	Falcarinol	Falcarindiol
1	*Anthriscus cerefolium* (L.) Hoffm., garden chervil	1	<0.7	≤1	≤0.4	160[b]	40
	Pimpinella major (L.) Hud., greater burnet saxifrage	1	≤0.5	20	60[b]	<20	<30
	Foeniculum vulgare Miller, fennel	3	≤0.4	3	0.3	40	50
	Anethum graveolens L., dill	≤0.4	≤0.7	≤0.7	≤0.3	100	180[b]
	Carum carvi L., caraway	1	≤0.6	4	3	90	5,300[c]
	Pastinaca sativa ssp. *sativa* L. cv. Halblange, parsnip	1	≤0.7	80,000[c]	23,000[c]	≤80[d]	<30
	Daucus carota L. spp. *sativus* cv. Danvers, carrot	300[c]	30[b]	≤1	≤1	110	120[b]
2	*Apium graveolens* L. var. *rapaceum* cv. Balder, celery root	7	<1	2,400[c]	5,600[c]	<17	<30
	Aegopodium podagraria L., ground elder	2	<0.3	12	9	500[b]	4,300[c]
	Petroselinum crispum (Miller) A. W. Hill, parsley	5	(1)[e]	400[c]	5,600[c]	<4	14
	Daucus carota L. spp. *sativus* cv. Danvers carrot	1,000[c]	30	4	≤0.6	40	100
	Daucus carota L. ssp. *sativus* cv. Sytan, carrot	3,700[c]	500[c]	1	6	80	200[b]
	Conium maculatum L., hemlock	<3[c]	<6[c]	9,600[c]	2,600[c]	2,100[c]	300[c]
	Heracleum sphondylium L., hogweed	<4[c]	<5[c]	4,800[c]	2,300[c]	400[c]	5,200[c]
	Estimated threshold for stimulation when applied as pure compound to filter paper leaf of 300 mm^2 total surface area (Städler and Buser, 1984)	1 mg	1 mg	1 mg	1 mg	(≤30 μg)	100 ng

[a]Amounts in ng/gle.
<, no signal three times greater than noise detected (detection threshold); ≤, signal three times greater than noise detected (SIM).
[b]Values are calculated from SIM data, but verified by complete mass spectra.
[c]Values are calculated from total ion counts (TIC), otherwise the values are obtained from SIM data.
[d]Maximal value, coelution with isobergapten, which has a small signal at m/z 159.
[e]Expected value according to regression of *t*-methylisoeugenol on *t*-asarone because of coelution with apiole.

Foliar Extracts. Extracts were prepared about 60 days (*Anethum graveolens* and *Anthriscus cerefolium*), 100 days (set 2), and 130 days (set 1) after sowing and two to four days after completion of the respective multiple choice oviposition assays with the intact leaves. We adopted a microwave-assisted extraction procedure (Paré et al., 1991) with hexane as a solvent that does not absorb radiation. This method produced extracts from carrot leaves that were highly effective in stimulating egg-laying in the carrot fly (Degen et al., 1999) and was superior to the dichloromethane dippings previously used to isolate oviposition stimulants (Städler and Buser, 1984; Städler et al., 1990). Cut, but otherwise undamaged, leaves (100 g each per plant species) were covered with 600 ml hexane (Fluka puriss. p.a.) in a 100-ml beaker, which was topped with a Petri dish to prevent solvent vapors from entering the oven body. Half a minute after the first contact of the foliage with the solvent, the beaker was placed in a microwave oven (Panasonic NN-6807) run for 60 sec at a power of 700 W. The heating of the plant material caused the temperature of the hexane to rise to 47–53°C. Afterwards, the solvent was immediately decanted into another beaker, so that the foliage was immersed on the whole for about 120 sec. The resulting solution was dried with Na_2SO_4, filtered (Schleicher & Schuell No. 595 1/2 folded paper filter), and concentrated on a rotary evaporator at 30°C and 240 mbar to 40 ml, i.e., 2.5 g leaf equivalents/ml (2.5 gle/ml).

Purification. Each crude extract was subjected to fractionation on silica gel according to Städler et al. (1990). A 16-ml aliquot corresponding to 40 gle was concentrated to ≈1 ml under a stream of N_2, transferred with a Pasteur pipett onto the column (75 × 11 mm, silicagel 70–230 mesh, Merck Kieselgel 60), and eluted successively with 20-ml portions of 100% hexane (Merck zur Rückstandsanalyse; fraction 1), 5% diethyl ether (Fluka puriss; stabilized with 0.0005% 2,6-di-*tert*-butyl-*p*-cresol) in hexane (fraction 2), twice 100% diethyl ether (fraction 3.1 and 3.2), and 100% methanol (Merck pro analysi; fraction 4). The diethyl ether fraction contained the previously identified oviposition stimulants (Städler and Buser, 1984) in a study which used dichloromethane surface extracts (Städler et al., 1990) and, therefore, the corresponding fraction 3.1 was chosen for the GC-MS analysis. All solutions were stored in a freezer at −20°C before further use.

Semiquantitative GC-MS Analysis of Fraction 3.1. Diethyl ether was removed from 20-gle aliquots of fraction 3.1 and replaced with ethyl acetate to reach a final concentration of 2 gle/ml. Aliquots of 1 μl of these solutions corresponding to 0.002 gle were on-column injected into a GC-MS.

GC-MS analysis was performed with a VG Tribrid mass spectrometer (VG Fisons, Manchester, England). The ion source was operated in the electron ionization mode (EI, 70 eV, 180°C). Full-scan mass spectra (m/z 35-435; 1.05 sec/scan; mass resolution $M/\Delta M$ = 500) were recorded. For quantification of particular target compounds present in low amounts, analysis also was carried

TABLE 2. IONS USED FOR SELECTED ION MONITORING (SIM), OTHER IMPORTANT IONS, AND ELUTION TEMPERATURES FOR SIX OVIPOSITION STIMULANTS

Compound	*m/z*		Elution temperature (°C)
	Quantitation ion	Other important ions	
trans-Methyl-isoeugenol	178 (M^+)	163, 147, 135, 115, 107	171
trans-Asarone	208 (M^+)	193, 165, 150	182
Xanthotoxin	216 (M^+)	201, 188, 173, 145	212
Bergapten	216 (M^+)	201, 188, 173, 145	213.5
Falcarinol	159	244 (M^+) very weak, 229, 173	210
Falcarindiol	157	260 (M^+), 242, 171, 129, 128, 115	225

out with selected-ion-monitoring (SIM) by using the molecular ions (*trans*-methylisoeugenol, *trans*-asarone, bergapten, xanthotoxin) or a major fragment ion (falcarindiol, falcarinol; see Table 2). A lock mass of *m/z* 207.033 from the silicon bleed of the HRGC column was used in SIM.

The analyses were carried out with a 24-m OV1 fused silica HRGC column (0.25 mm ID) that was temperature programmed as follows: 70°C, 2 min isothermal, 20°C/min to 160°C, then at 5°C/min to 280°C, followed by an isothermal hold at this temperature. Data acquisition started when the column reached 160°C. The compounds of interest eluted between ~2 min (methylisoeugenol) and 13 min (falcarindiol).

The amount of analyte was determined from peak height measurements relative to known amounts injected (SIM or total ion data). Concentrations of the polyacetylene standards were not reliably known, as signals obtained for falcarindiol and falcarinol were lower than expected when compared to equal amounts of furanocoumarins. Thus, we adjusted the originally calculated amounts for these two compounds with correction factors, assuming that identical quantities of polyacetylenes and furanocoumarins result in identical peak heights (total ion data). Major additional compounds present in fraction 3.1 were identified according to their mass spectra and retention times, when a standard was available, or tentatively from the interpretation of the mass spectra alone. The amounts were estimated by comparing the peak height to the peak heights of standards or of the key compounds belonging to the same or a related compound class.

Insects and Oviposition Assays. Flies for the oviposition experiments were obtained from a laboratory culture reared according to Städler (1971) and were kept in a controlled-environment chamber at 21 ± 1°C, 70–80% relative humidity, and 16 : 8-hr light–dark regime. Oviposition preference hierarchies were established separately for two sets of eight plants, each including the standard

carrot cultivar Danvers and a nonhost. Multiple choice assays were conducted in cubic screen cages (0.34 m^3) containing 100–400 individual flies (males and females). Eight oviposition devices (Städler, 1971) equipped with foliage or with surrogate leaves were arranged in a circle around an apple seedling (resting place) in the center of the cage. Surrogate leaves, made of green cardboard and coated with a thin layer of paraffin (model "three-dimensional hogweed," see Degen and Städler, 1997), were sprayed with 4 gle of the respective fraction 3.1. The positions of the eight treatments were changed for each of the eight experimental periods, which usually lasted one day (randomized complete block design).

Statistics. Egg counts were tested for a treatment effect (plant species) with the Friedman test. Multiple comparisons among treatments were made following the method based on rank sum differences given by Conover (1980). To establish the functional relationship among the contents of oviposition stimulants and the stimulatory activities of the respective fractions 3.1, we performed a stepwise multiple regression (forward; F-to-enter = 4.000; F-to-remove = 3.996). Since decreasing the concentration of crude hexane extracts by a constant proportion produced over a broad range an approximately linear reduction in oviposition (see Figure 2 in Degen et al., 1999), log-transformed concentrations of the compound groups (see Figure 3 below) were used as independent variables. Along with these single terms, we also included two-way interaction terms in the regression model, i.e., the products of two independent variables each. The dependent variable was calculated as follows:

$$\text{relative oviposition} = \frac{\text{mean \% eggs laid with test plant}}{\text{mean \% eggs laid with carrot cv. Danvers}} \times 100$$

RESULTS

The fractionation procedure resulted in no obvious loss of stimulatory activity, since a combination of all fractions did not elicit significantly less oviposition than the crude extract in multiple choice assays carried out with four selected examples (standard carrot *Daucus carota sativus* cv. Danvers of set 1 and 2, *Conium maculatum*, *Carum carvi*; data given in Degen and Städler, 1998). These experiments also showed that the stimulatory activity was confined to fraction 3.1 and fraction 4. The low acceptability of surrogate leaves treated with the additional diethyl ether fraction 3.2 indicated that there was no substantial carryover of stimulants from fraction 3.1. Hence, other compounds yet unknown must be responsible for the stimulatory activity of fraction 4.

The quantities of oviposition stimulants and related secondary compounds (Figure 1) in fraction 3.1 varied widely among host-plant species (Tables 1 and

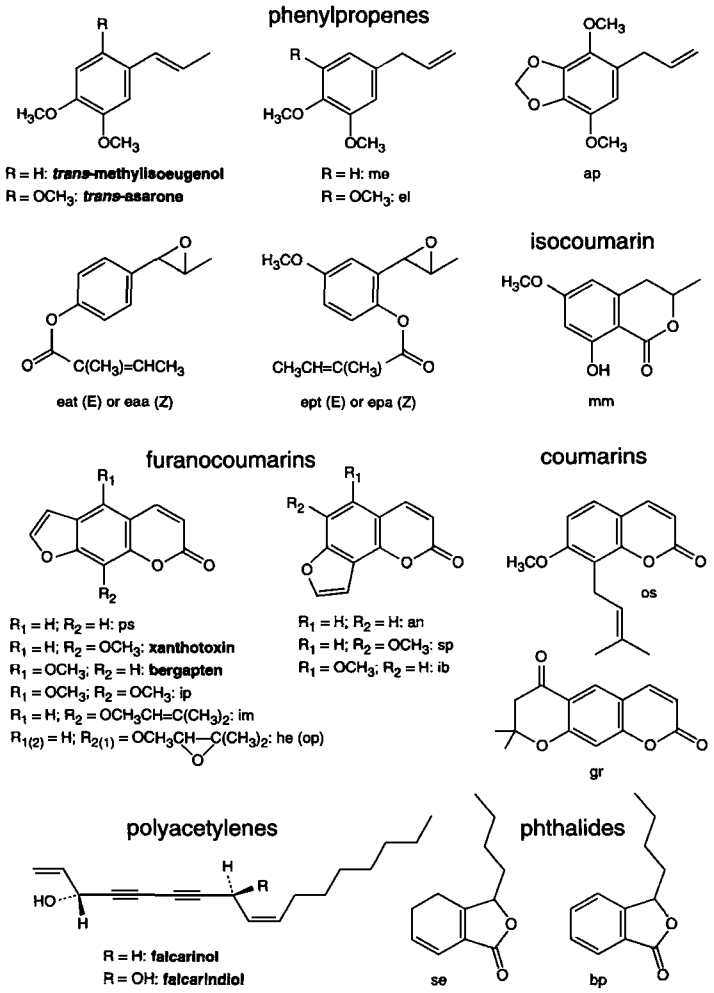

FIG. 1. Structural formulae of six oviposition stimulants (key compounds in bold letters) and of related compounds detected in various host-plant species (for abbreviations see Table 3).

3, Figures 2 and 3C), while plants were similar to each other with respect to major wax components such as long-chain fatty alcohols and sterols. This is exemplified by the chromatograms depicted in Figure 2.

Concentrations of the six oviposition stimulants (on a log scale) were correlated within, but not among the compound groups, i.e., high amounts of

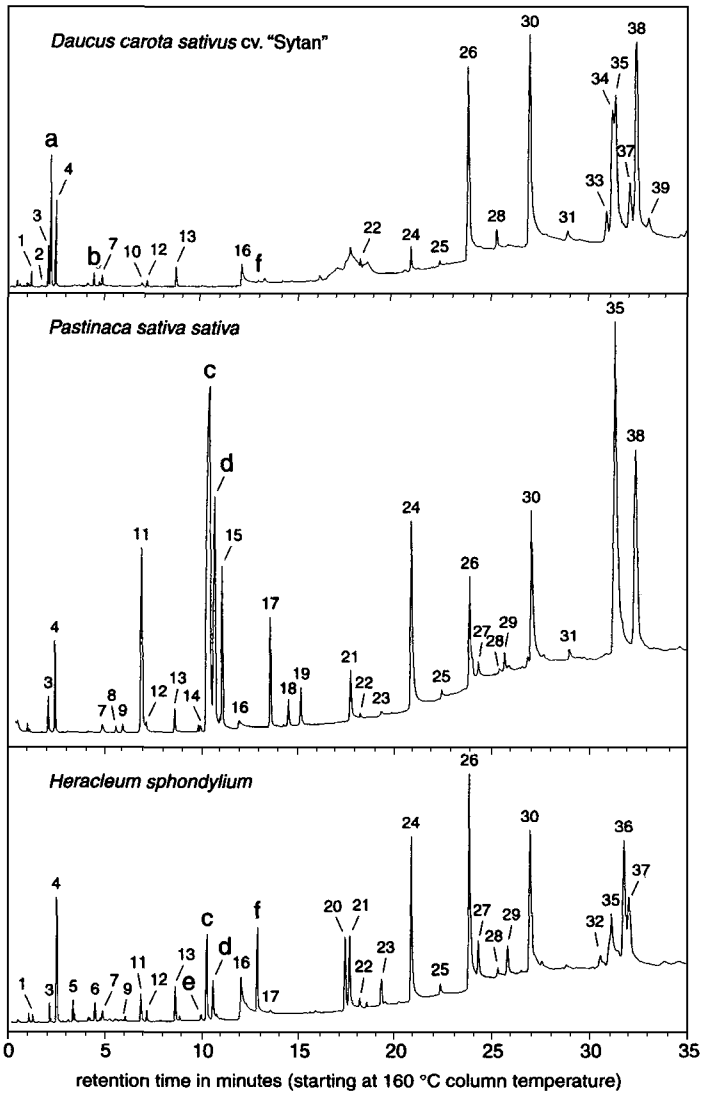

FIG. 2. Chromatograms from total ion scans of fraction 3.1 of three selected host-plant species that differ markedly in their pattern of oviposition stimulants (key compounds). Key compounds: a = *trans*-methylisoeugenol; b = *trans*-asarone; c = xanthotoxin; d = bergapten; e = falcarinol; f = falcarindiol. Additional components: 1 = methyleugenol; 2 = *cis*-methylisoeugenol; 6 = apiol; 9 = angelicin; 10 = 6-methoxymellein, 11 = psoralen; 14 = sphondin or isobergapten; 15 = 5-dodecyldihydro-2(3H)-furanone; 16 = linolenic acid; 17 = isopimpinellin; 19 = imperatorin; 20 = oxypeucedanin or heraclenin; 21 = *n*-docosanol; 23 = *n*-tricosanol; 24 = *n*-tetracosanol; 25 = *n*-pentacosanol; 26 = *n*-hexacosanol; 28 =

trans-methylisoeugenol were detected together with relatively high amounts of *trans*-asarone ($r = 0.91$; Fisher's r to z P-value <0.0001; $N = 13$; without *Petroselinum crispum*). The same was found for the two furanocoumarins ($r = 0.95$; $P < 0.0001$; $N = 14$) and the two polyacetylenes ($r = 0.63$; $P = 0.013$; $N = 14$). When the species with amounts below detection threshold (values with < in Table 1) are excluded from the calculation of r, the correlation is still significant for the phenylpropenes ($r = 0.96$; $P < 0.0001$; $N = 8$), but no longer for the polyacetylenes ($r = 0.40$; $P = 0.26$; $N = 10$) because the ratio of falcarinol to falcarindiol was more variable. Nevertheless, the summation of the amounts of stimulants belonging to the same compound class as shown in Figure 3C seems to be justified for a simplified comparison of the stimulant composition in the various host plants.

We found essentially four major patterns in the distribution of stimulants: (1) High levels of furanocoumarins only were observed with *Pastinaca sativa*, *Apium graveolens*, and *Petroselinum crispum*. (2) High contents of polyacetylenes only were found with *Carum carvi* and *Aegopodium podagraria*. (3) The phenylpropenes *trans*-methylisoeugenol and *trans*-asarone were present in substantial amounts only in the three carrot extracts together with medium amounts of polyacetylenes. (4) High quantities of both furanocoumarins and polyacetylenes were detected in *Conium maculatum* and *Heracleum sphondylium*. The remaining species contained at best moderate amounts of a single compound class (e.g., furanocoumarins in *Pimpinella major*, polyacetylenes in *Anethum graveolens* and *Anthriscus cerefolium*). There was no example with a combination of both phenylpropenes (i.e., *t*-methylisoeugenol, *t*-asarone) and furanocoumarins or of all three compound classes occurring together in high quantities.

In Figure 3, we compared the relative numbers or eggs deposited by carrot flies underneath real leaves and surrogates treated with fraction 3.1 to the distribution of the six oviposition stimulants in the respective plant species. Leaf models treated with fractions containing high levels of only a single compound class received only moderate numbers of eggs (e.g., polyacetylenes in *Carum carvi* and *Aegopodium podagraria*, furanocoumarins in *Pastinaca sativa*). Strong

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heptacosanol; 29 = β-tocopherol; 30 = *n*-octacosanol; 31 = *n*-nonacosanol. Contaminants: 3 = ionox; 4 = butylhydroxytoluol (BHT, antioxidant); 12 = diisobutylphthalate (DiBP); 13 = dibutylphthalate (DBP); 22 = dioctylphthalate (DOP). Unidentified compounds: 5 = oxygenated sesquiterpene; 7 = m/z 224 (M^+), 208, 193, 123; 8 = m/z 188 (M^+), 160, 146, 18 = m/z 280 (M^+?); 27 = m/z 402 (M^+), "desmethyl-tocopherol"; 32 = m/z 414 (m^+), sterol; 33 = m/z 426 (M^+), sterol; 34 = m/z 426 (M^+), sterol; 35 = m/z 426 (M^+), sterol (amyrin); 36 = m/z 414 (M^+), sterol; 37 = m/z 426 (M^+), sterol; 38 = m/z 426 (M^+), sterol (amyrin); 39 = m/z 426 (M^+), sterol.

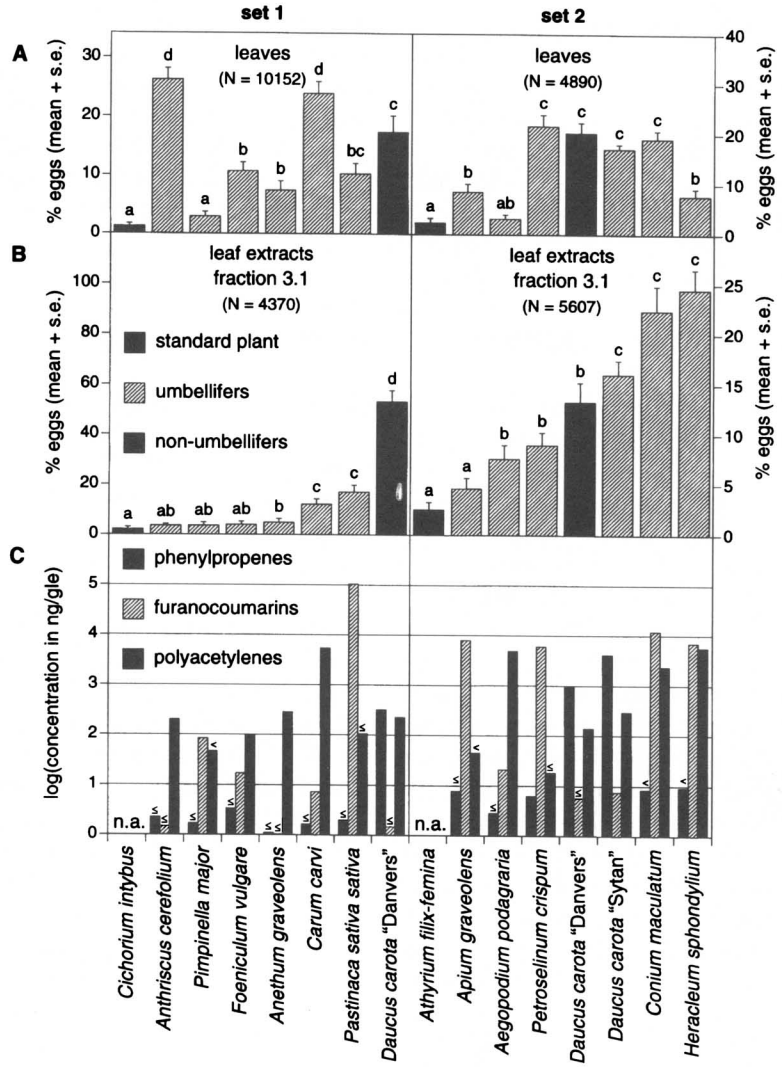

FIG. 3. A and B: Oviposition of carrot flies underneath real leaves (A) and surrogate leaves treated with 4 gle of fraction 3.1 of the respective leaf surface extracts (B). Two sets of eight plants each including the standard carrot cultivar Danvers were tested separately in multiple choice assays. The numbers of eggs are expressed as percent total oviposition during an experimental period. The y axes are adjusted so that columns of the standard plant are of equal height with both sets of host species. The treatment had a significant effect (Friedman test: $P < 0.0001$) in all four experiments. Means accompanied by the same letter are not significantly different at the 5% level. Eight replicates per treatment in each experiment. C: Amounts (logarithmic scale) of oviposition stimulants detected

ovipositional responses were elicited only when two compound classes were present in medium to high amounts (e.g., phenylpropenes and polyacetylenes in *Daucus carota*, furanocoumarins and polyacetylenes in *Heracleum sphondylium* and *Conium maculatum*).

With both sets of plant species, the products log(phenylpropenes) * log(polyacetylenes) and log(furanocoumarins) * log(polyacetylenes) accounted for a high percentage of the variation in stimulatory activity of fractions 3.1, and no other variable was entered into the stepwise regression model (Table 4). With set 1, however, the effect of the second two-way interaction term was not significant at the 5% level. Moreover, in this case, the single term log(phenylpropenes) explained almost as much variation in a simple regression model (adjusted R^2 = 0.86; P = 0.002) as the two interaction terms in the stepwise regression model (adjusted R^2 = 0.93; P = 0.002). Since in all plant species examined at least one compound group was present only in amounts that are probably negligible, we refrained from including a three-way interaction term, i.e., the product of all three compound groups. With set 2, this three-way interaction would have been the only term entered into the stepwise regression model (adjusted R^2 = 0.78; P = 0.005), whereas with set 1 the model would have been left unchanged.

The preference hierarchies obtained for real leaves and for fractions 3.1 do not agree well (correlations using mean arcsine-transformed percentage of eggs; set 1: r = 0.40, P = 0.34; set 2: r = 0.54, P = 0.17). This is illustrated by the low stimulatory activity of fraction 3.1 in *Anthriscus cerefolium*, a highly preferred host plant (Figure 3).

Additional compounds present in fraction 3.1 were identified whenever possible (Table 3), but were not screened systematically by selected ion monitoring (SIM). Traces of *cis*-methylisoeugenol accompanied the *trans* isomer in the carrot cultivar Sytan. On the other hand, the occurrence of the allyl-isomer methyleugenol (e.g., in *Anthriscus cerefolium*), of related allylbenzenes (e.g., apiol in *Petroselinum crispum*, elemicine in *Conium maculatum*), or of structurally related compounds (epoxy-pseudoisoeugenol ester and epoxy-anol ester in *Pimpinella major*) did not seem to be linked to the contents of *trans*-methylisoeugenol or *trans*-asarone. By contrast, the incidence of further linear furanocoumarins (e.g., psoralen, isopimpinellin) was restricted to plants that contained considerable amounts of xanthotoxin and bergapten. Angular furanocoumarins were seen only in *Pastinaca sativa* and *Heracleum sphon-*

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in the corresponding fraction 3.1 summarized for compound classes (phenylpropenes = *t*-methylisoeugenol and *t*-asarone; furanocoumarins = xanthotoxin and bergapten; polyacetylenes = falcarinol and falcarindiol). n.a. = not analyzed. ≤ = maximal amount, signal detected with SIM; < = detection threshold, no signal three times greater than noise detected.

TABLE 3. FURTHER RELATED COMPOUNDS IDENTIFIED IN FRACTION 3.1[a]

		Phenylpropanoids								Furanocoumarins					(Iso)coumarins			Phthalides	
Set	Compounds	*c*-mi	*t*-ie	me	el	ap	eat/ eaa	ept/ epa	ps	ip	im	op/ he	an	sp/ ib	mm	os	gr	se	bp
	Identification according to	b[b]	a	a	b	a	b	b	a	a	a	b	a	b	b	a	b	a	a
1	*Anthriscus cerefolium*	–	+	++	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Pimpinella major	–	–	–	–	–	+++[d]	++	–	–	–	–		–	++	–	–	–	–
	Foeniculum vulgare	–	–	±[c]	–	–	–	–	–	–	–	–	–	–	–	–	++	±	
	Anethum graveolens	–	–	–	–	–[e]	–	–	–	–	–	–	–	–	–	–	++	+	–
	Pastinaca sativa	–	–	–	–	–	–	–	+++	++	++	–	+	+	–	–	–	–	–
2	*Apium graveolens*	–	–	–	–	–	–	–	–	++	–	–	–	–	–	–	–	+++	++
	Petroselinum crispum	–	–	–	+	++	–	–	++	+	–	++	–	–	–	–	++	+	–
	Daucus carota Danvers	–		–	–									–	±[c]	–			

Daucus carota																		
Sytan	±	±	+	±[c]		–	–	–	–	–	–	–	–	+	–	–		–
Conium																		
maculatum	–	–	+	+	–	–	–	++	++	–	–	–	–		+	–	(±[f])	–
Heracleum																		
sphondylium	–	–	+	±	++	–	–	++	+	–	++	±	–		–	–	(±[f])	
Stimulatory																		
activity	+	–[g]	–		–			–	+	+				–	+		–	–
Amount[h]	10 mg	10 mg	10 mg		10 mg			1 mg	30 μg	2 mg				1 mg	300 μg			

[a]Phenylpropanoids: *c*-mi = *cis*-methylisoeugenol; *t*-ie = *trans*-isoeugenol; me = methyleugenol; el = elemicin; ap = apiole; eat/eaa = epoxy-anol tiglate or angelicate (isomers); ept/epa = epoxy-pseudoisoeugenol tiglate or angelicate (isomers). Furanocoumarins: ps = psoralen; ip = isopimpinellin; im = imperatorin; op/he = oxypeucedanin or heraclenin (isomers); an = angelicin; sp/ib = sphondin or isobergapten (isomers). Isocoumarins and coumarins: mm = 6-methoxymellein; os = osthol; gr = graveolone. Phthalides: se = sedanenolide; bp = butylphthalide. Approximate concentrations: – = <10 ng/gle; ± = 10–100 ng/gle; + = 100–1000 ng/gle; ++ = 1–10 μg/gle; +++ = 10–100 μg/gle. None of these additional compounds were detected in *Carum carvi* and *Daucus carota* Danvers (set 1) and in *Aegopodium podagraria* (set 2).

[b]a, Retention time and mass fragmentation (standard compound injected); b, mass fragmentation.

[c]SIM.

[d]Plus small amounts of epoxy-anol 2-methylbutyrate (100–1000 ng/gle).

[e]Traces.

[f]Possible carryover from preceding injection of celery root fraction.

[g]Inhibitory effect on oviposition.

[h]Minimal amount tested that was active (+) or maximal amount tested that was inactive (–) when applied to filter paper leaf of 300 mm^2 total surface area (E. Städler, unpublished).

TABLE 4. STEPWISE MULTIPLE REGRESSION FROM LOGARITHMIC CONCENTRATIONS (IN ng/gle) OF OVIPOSITION STIMULANTS (SUMMARIZED FOR COMPOUND CLASSES AS SHOWN IN FIGURE 3) ON STIMULATORY ACTIVITY OF FRACTION 3.1[a]

Set	Independent variables	Coefficient	SE	Std. Coeff.	*t* value	*P* value (*t*)	R^2 (adjust.)	*F* value	*P* value (*F*)
1	Entered in model						0.95 (0.93)	38.90	0.002
	Step 1: log(PHE) * log(POL)	17.09	1.94	1.02	8.81	0.001			
	Step 2: log(FUR) * log(POL)	2.26	1.12	0.23	2.02	0.11			
	Intercept	−3.53	5.84		−0.60	0.58			
	Not entered in model		*Partial correlation*						
	log (PHE)			−0.63	−1.41	0.25			
	log (FUR)			−0.38	−0.71	0.53			
	log (POL)			0.51	1.03	0.38			
	log (PHE) * log (FUR)			−0.60	−1.30	0.28			
2	Entered in model		*SE*	*Std. coeff.*			0.90 (0.85)	18.31	0.01
	Step 1: log(FUR) * log(POL)	10.07	1.82	0.93	5.53	0.005			
	Step 2: log(PHE) * log(POL)	13.83	3.24	0.72	4.27	0.013			
	Intercept	−16.26	21.87		−0.74	0.50			
	Not entered in model		*Partial correlation*						
	log (PHE)			−0.03	−0.05	0.96			
	log (FUR)			−0.20	−0.36	0.74			
	log (POL)			0.10	0.17	0.87			
	log (PHE) * log (FUR)			−0.25	−0.45	0.68			

[a]Dependent variable: percent oviposition relative to standard carrot cultivar. PHE = phenylpropenes = *trans*-methylisoeugenol + *trans*-asarone; FUR = furanocoumarins = xanthotoxin + bergapten; POL = polyacetylenes = falcarinol + falcarindiol.

dylium. Osthol, a substituted coumarin identified earlier as an oviposition stimulant in carrot extracts (Städler and Buser, 1984), was detected only in *Conium maculatum*. *Anethum graveolens*, *Foeniculum vulgare*, and *Petroselinum crispum* contained substantial amounts of graveolone, another coumarin, which was first isolated and described from dill plants (Aplin and Page, 1967). The phytoalexin 6-methoxymellein, an isocoumarin, was found in carrots, *Daucus carota*, and in *Pimpinella major*. *Apium graveolens* was characterized by high levels of phthalides. Falcarinone, a further polyacetylene, was present in some of the samples in trace amounts detectable only by SIM, but not verifiable by full-scan mass spectra.

DISCUSSION

Plant extracts have only rarely been analyzed by GC-MS for falcarinol and falcarindiol, which are known to decompose easily (e.g., Städler and Buser, 1984; Mercier et al., 1993). Our study shows that a quantification of polyacetylenes by this method is feasible with certain reservations, e.g., possible nonlinearity due to irreversible adsorption (Yates and England, 1982) and a higher detection threshold than for other compounds. Taking these difficulties into consideration, the amounts given in Table 1 probably are minimal values. In any case, the absolute concentrations should be compared among compound classes only with caution, since a standard with a precisely known concentration was not available for polyacetylenes. On the other hand, relative differences in amounts detected among plant species are fairly reliable, especially because variation in contents ranged more than 2–5 orders of magnitude. Ultimately, the latter was more crucial. The data given in Table 1 originate from single GC runs in most cases, yet standard errors of means from injections of two independent samples proved to be small in an earlier study using the same method (Städler et al., 1990).

Although there is a wealth of studies dealing with secondary plant compounds in umbellifers, mostly using roots or seeds as a source for the extractions, relatively few have included several umbelliferous genera belonging to different tribes (e.g., Berenbaum, 1981; Ceska et al., 1987). Here, we present data on leaf surface contents of three characteristic compound classes from 12 species raised under the same or similar environmental conditions. If we regard components in concentrations below 10 ng/gle as quasi-absent, the frequency of compound classes declines in the following order: polyacetylenes, furanocoumarins, propenylbenzenes. The C17 polyacetylenes were recorded earlier from all plant species examined in this study, with the possible exception of *Anthriscus cerefolium* and *Foeniculum vulgare* (Bohlmann, 1971; Hegnauer, 1973). More recent investigations revealed the occurrence of falcarinol and fal-

carindiol in *Aegopodium podagaria* (Kemp, 1978) and in *Daucus carota* (e.g., Lund and Bruemmer, 1991; Mercier et al., 1993). Edible parsley root *Petroselinum crispum* ssp. *tuberosum* is a rich source of both compounds, whereas ssp. *crispum* yields only minor amounts (Nitz et al., 1990). While the falcarinone-type polyacetylenes apparently are more universally distributed among umbellifers than the other stimulants, they also seem to be most specific to Apiaceae (Städler, 1986), although they also occur in the closely related Araliaceae (Hansen and Boll, 1986; Boll and Hansen, 1987) and in the Asteraceae (Hegnauer, 1971). Falcarindiol, surprisingly, also has been reported as a phytoalexin in tomatoes (De Wit and Kodde, 1981). The distribution of furanocoumarins is in fairly good agreement with earlier investigations of leaf and seed contents (Berenbaum, 1981; Ceska et al., 1987). According to Harborne (1971), phenylpropenes with a 2,4,5-substitution pattern, such as in asarone, are confined to *Daucus carota*. Our results support this notion.

The production of allelochemicals in plant tissues is affected by developmental and environmental factors. For example, furanocoumarin content in umbelliferous species varies with season (Zobel and Brown, 1990) and with light and nutrition (Zangerl and Berenbaum, 1987). Likewise, changes in furanocoumarin content may arise in response to stress by UV irradiation or low temperatures (Beier and Oertli, 1983), to pathogen attack (Heath-Pagliuso et al., 1992), and to insect feeding (Zangerl, 1990). Therefore, it is conceivable that the damage inflicted by the hail upon the young test plants in our study may have influenced the levels of secondary substances under investigation. However, this is not relevant here since the main objective was to demonstrate the effect of the semiochemicals on ovipositional behavior and not to quantify absolute contents of semiochemicals in host plants. Even acknowledging pronounced variability in response to environmental factors, the general pattern in semiochemical distribution shown by particular plant species is probably quite constant, as both constitutive and induced levels can be expected to be under genetic control (Zangerl and Berenbaum, 1990). For example, linear furanocoumarins were found in foliage of *Petroselinum crispum* together with graveolone in proportions similar to those observed in our study (Beier et al., 1994). Likewise, the partially resistant carrot cultivar Sytan invariably exhibited higher concentrations of *trans*-methylisoeugenol and *trans*-asarone than the susceptible cultivar Danvers in this and two previous analyses (Guerin and Städler, 1984; Städler et al., 1990). Varietal differences in oviposition stimulants can apparently also be observed with *Apium graveolens*: the polyacetylene content of the cultivar Balder was below detection threshold in this study, whereas concentrations in the cultivar Tropa were higher than those in carrots (Städler et al., 1990). This deficiency may be specific to the cultivar Balder and may be the reason for its relatively low acceptability compared to other celery root cultivars.

According to the stepwise multiple regression analysis, the variation in

stimulatory activity of fractions 3.1 is better explained by a multiplicative than by an additive action of oviposition stimulants belonging to different compound groups. This result must be interpreted with caution, however, as some assumptions and requirements of the regression model may not have been properly met by our data. In a few cases, concentrations of stimulants were close to or below the detection threshold and hence were not accurately known. This is unlikely to have biased the outcome of the stepwise regression. The low number of observations, seven per plant set, is another drawback. It would not have been correct statistically to increase the sample size by combining both sets because the values within one set are not independent of each other, an inherent problem of multiple-choice tests. The effect of a particular treatment seemed to depend on the range of other treatments present, as evidenced by the higher proportion of eggs (relative to the standard plant) deposited below surrogate leaves treated with the nonhost fraction in set 2. The crude hexane extracts of both nonhost species did not differ in stimulatory activity when tested together in a multiple-choice oviposition assay, and the same applies to the two crude extracts of the carrot cultivar Danvers (see Degen and Städler, 1998). As the two latter extracts did not contain exactly the same quantities of oviposition stimulants, they cannot be regarded as standards in a strict sense, but variation between them was only minor compared to that among other host taxa. Nevertheless, the percentages for plant species are not directly comparable between sets, even when related to the standard plant. For example, *Carum carvi* significantly differed from the standard carrot in set 1, whereas in set 2 the difference between the standard carrot and *Aegopodium podagraria* with a pattern of stimulants similar to the pattern of *Carum carvi* was not significant. (However, experience shows that the ranking of treatments is less affected by experimental design, i.e., the range of other treatments included, than are the percentage). By chance, most plants chosen for set 1 contained fewer oviposition stimulants and, consequently, activities of fractions 3.1 relative to the standard were generally lower than in set 2. This difference in profiles of stimulants may explain in part why the two regression models yielded somewhat divergent results. Collinearity among single and interaction terms constitutes another complication. Notably with plant set 1, phenylpropenes alone and the phenylpropenes * polyacetylenes interaction were almost equivalent in explaining the observed variation, thus making it hard to decide which of the factors actually matters. However, given that the detected polyacetylene concentrations represent minimal values, the fact that the polyacetylenes, in particular falcarindiol, have a considerably lower activity threshold than phenylpropenes (when tested as pure compounds) makes the activity differences among fractions 3.1 in set 1 appear difficult to understand, unless an interaction among polyacetylenes and phenylpropenes is inferred. Finally, another disturbing feature of the model is the wrong prediction that pure stimulatory compounds exert no influence on egg-laying in the absence of other oviposition stimulants. While

the regression models give some insight into the causative relationship among the contents of stimulants and oviposition, their predictive value is limited. The results should be verified by a full factorial oviposition choice experiment including all possible combinations of pure compounds belonging to the three groups. This could help to unravel any independent effect of each factor and their interactions.

In conclusion, the six surveyed oviposition stimulants seem to account for a major part of the variation in the stimulatory activity of fraction 3.1, and they may do so in a synergistic manner. By surveying blends of oviposition stimulants actually occurring in host plants, we confirmed earlier findings obtained by testing mixtures of pure compounds (Städler and Buser, 1984). Some unexplained differences in the numbers of eggs received (e.g. among *Apium graveolens* and *Petroselinum crispum*) may be due to additional components of known or unknown activity (Table 3), to unidentified constituents, or to early eluting (T_e < 160°C) compounds not analyzed (e.g., monoterpenes, further phenylpropenes). The nonpreference of carrot flies for fractions of *Anethum graveolens*, *Foeniculum vulgare*, and *Pimpinella major* provides circumstantial evidence for the lack of stimulatory activity of graveolone and the pseudoisoeugenols, respectively, which have not yet been tested as pure compounds. It should also be kept in mind that the relationship among the amounts of semiochemicals (on a logarithmic scale) and the behavioral response need not necessarily be linear, as is assumed for the stepwise regression model. There might be optimal concentrations for particular stimulants or optimal ratios among different compounds.

The oviposition stimulants quantified in this study are perceived by olfactory sensilla on the carrot fly's antennae, but not by contact chemoreceptors of the tarsal sensilla (Städler and Roessingh, 1991). The synergistic effect of the compounds implies that there are probably at least two distinct receptors for the stimulants. Since none of the plants exhibited high levels of both phenylpropenes and furanocoumarins, we do not know whether these two compound groups also synergize in eliciting egg-laying and are bound to different receptors. The apparently higher ovipositional response to phenylpropenes than to furanocoumarins could be due to differences in receptor sensitivity. Alternatively, it could be an artifact caused by an interaction of the paraffin covering the surrogate leaves and the oviposition stimulants, which potentially leads to varying release rates for different compounds.

The preference hierarchy of the carrot fly established with foliage in the laboratory is fairly representative for differential susceptibility of the respective plants to carrot fly attack in the field (Hardman et al., 1990; Degen, Städler, and Ellis, unpublished). While the known oviposition stimulants appear to be responsible for the varying activities of fraction 3.1, they do not properly reflect the differences in acceptability observed among real leaves. Hence, we are still far away from a complete understanding of the factors governing host choice of the

carrot fly. Further stimulatory compounds eluting into the methanol fraction of the hexane extract seem to play an important role. Moreover, it has been found that polar deterrents present in host plants potentially also affect oviposition preferences (Degen and Städler, 1998). As long as these additional semiochemicals remain unidentified, it is difficult to assess the full ecological significance of the stimulants dealt with in this paper. Although they may be involved in mediating host-plant preference in concert with other stimulatory and inhibitory compounds, their main role may lie in being signals that promote host-plant recognition in a broad sense. The present study focused on factors responsible for variation in antixenotic resistance among hosts. Yet, carrot flies may be more often confronted with discrimination among nonhosts (nonumbelliferous plants) and hosts (most umbellifers) under natural conditions than with weighing alternative host-plant species.

Future research projects should concentrate on the identification of additional behaviorally active compounds as well as on the sensory physiology underlying host recognition and acceptance. The existing evidence suggests that the carrot fly is another example of an insect whose host choice is based on the integration of a complex of chemical and nonchemical plant stimuli (Städler, 1992).

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EXTRACTING OVIPOSITION STIMULANTS FOR CARROT FLY FROM HOST-PLANT LEAVES

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Abstract—Extracts of carrot foliage obtained with various extraction methods were compared for effectiveness in stimulating oviposition in the carrot fly. In choice assays, surrogate leaves treated with a hexane surface extract produced with a new microwave-assisted procedure were almost as acceptable as real host leaves. The high stimulatory activity of this extract was attributable to the raised solvent temperature, since cold hexane extracts were much less stimulatory. The microwave extract elicited about twice as much oviposition as the previously used dichloromethane surface extracts and the diethyl ether fraction of an extract that was obtained by brief immersion of leaves into water near its boiling point. The ovipositional responses to crude methanol and hot water extracts were weak because of the presence of yet unidentified polar deterrent compounds. Total extracts of ground foliage (vacuum distillation and extraction with liquid carbon dioxide) had no net stimulatory effect on oviposition.

Key Words—*Psila rosae*, Diptera, Psilidae, *Daucus carota*, *Heracleum sphondylium*, Apiaceae, microwave-assisted extraction, leaf surface extracts, oviposition stimulants, oviposition deterrents.

INTRODUCTION

The carrot fly, *Psila rosae* (F.), is an oligophagous pest insect whose larvae feed on the roots of a wide range of cultivated (e.g., carrot, celeriac, parsley) and wild umbelliferous species (Ellis et al., 1992). The female flies per-

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form exploratory runs over the leaves of potential host plants and, when stimulated, move down the stem axis to deposit eggs in the soil near the plant's base (Bohlen, 1967). The first recorded oviposition stimulant for the carrot fly, *trans*-methylisoeugenol, was isolated from steam-distilled carrot leaves (Berüter and Städler, 1971). Later, Städler and Buser (1984) identified five additional stimulatory compounds in dichloromethane washings of undamaged carrot foliage. Yet, surrogate leaves treated with high concentrations of surface extracts elicited much less egg-laying than real leaves (Städler, 1977). Apart from deficiencies in the physical properties of the artificial leaves made of filter paper, this result may also reflect inadequate chemical stimuli originating from the extract. In support of this idea, previous investigations failed to demonstrate a correlation between the amounts of known oviposition stimulants (phenylpropenes, furanocoumarins, polyacetylenes) and antixenosis resistance of different cultivars (Visser and de Ponti, 1983; Guerin and Städler, 1984; Städler et al., 1990). This prompted us to reevaluate several standard extraction procedures (for a survey see Städler and Roessingh, 1991) together with a new microwave-assisted method (Paré et al., 1991). We directly compared the stimulatory activity of these extracts in oviposition choice assays using surrogate leaves that were optimized for foliar form, color, and surface characteristics (Degen and Städler, 1997a). This investigation is a further step towards the identification of all essential chemical stimuli that determine the host-plant preferences of ovipositing carrot flies.

METHODS AND MATERIALS

Extracts

We used foliage from carrot plants grown in a greenhouse (20 ± 2°C; 16-hr light per day, which comprised both natural light and sodium lamps to simulate daylight) at Rothamsted Experimental Station (UK) for the following three extracts:

Vacuum Distillation. Foliage was ground in a mortar in liquid N_2. This was extracted with 500 ml diethyl ether for 24 hr. The dark green extract was poured through filter paper (Whatman No. 1) and dried with $MgSO_4$. The filtered ether solution was rotary evaporated to about 20 ml. This concentrated extract was distilled under a vacuum of <0.05 mm Hg for 24 hr in an apparatus similar to that described by Pickett and Griffiths (1980). The distillate was transferred at a concentration of 20 gram leaf equivalents (gle)/ml into an ampoule sealed under N_2.

Carbon Dioxide. Leaves were crushed in liquid N_2 and the resultant ground mixture was extracted with liquefied CO_2 for 90 min at a pressure of 60 bar and a temperature of 11–19°C. Diethyl ether was added to the CO_2 vessel to obtain a solvent extract. This solution was dried with anhydrous $MgSO_4$, filtered, concentrated under N_2, and stored in a sealed ampoule.

Microwave-Assisted Extraction with Hexane. Entire foliage was placed in an extraction vessel, covered with hexane, and placed in a microwave oven that was run for 60 sec at 800 W power. Hexane itself does not absorb microwave radiation, but it is heated indirectly by the water-containing plant material (Paré et al., 1991). A distillation apparatus was placed on top of the extraction vessel to ensure that no solvent vapor could enter the oven body. The distillation column traversed the ceiling of the oven where an antenna and a choke were used to prevent microwaves from escaping. At present, special microwave apparatus is commercially available for extraction purposes.

To prepare the other extracts, we used leaves from plants grown outside in seed beds at the Swiss Federal Research Station in Wädenswil and foliage from wild hogweed plants collected locally. Cut, but otherwise undamaged, leaves were either held by the stems and dipped into beakers filled with solvent (water, methanol, dichloromethane) or totally submerged (hexane). The extraction methods differed in duration, solvent polarity, and temperature (Table 1):

Hot Water. Leaves were dipped for about 2 sec in a beaker filled with almost boiling distilled water (Zobel and Brown, 1988). A part of this extract was separated into water and diethyl ether fractions (by extracting it three times with diethyl ether).

Methanol. Leaves were successively immersed for 10 or 30 sec each in two beakers containing methanol. Between the dippings they were kept in the air for 5 sec. An aliquot of the 2×30 sec extract was evaporated to a smaller volume, taken up with water, and extracted five times with diethyl ether to obtain a water and diethyl ether fraction.

Dichloromethane. Leaves were washed twice for 30 sec with CH_2Cl_2 (Städler and Buser, 1984). During the time between dippings (5 sec), evaporation of the solvent cooled the leaves and consequently the solvent in the second breaker.

Hexane in Microwave Oven. Microwave-assisted extractions were carried out in a similar manner as described above. Foliage was covered with hexane in a 1000-ml breaker topped with a Petri dish to prevent the escape of hexane vapors. Half a minute after first contact with the solvent, the beaker was placed in an unmodified household microwave oven that was set up in a fume hood and run for 30 or 60 sec at a power of 700 or 900 W. Thereafter the solvent was immediately poured off. Leaves were in contact with the solvent for a total of approximately 90 and 120 sec, respectively. The temperature reached 47–53°C (with extractions lasting for 60 sec) just after the beaker had been taken out of the microwave oven.

Hot and Cold Hexane. For comparison with the microwave technique, two additional extracts were prepared by covering leaves in a beaker with cold (22°C) and heated (50–56°C) hexane, respectively for 120 sec in the fume hood.

The dichloromethane and hexane solutions were dried with anhydrous

TABLE 1. LEAF EXTRACTS TESTED IN OVIPOSITION EXPERIMENTS AND SOME EXTRACTION PARAMETERS[a]

Method	Plant species	Weight (g)	Solvent	Volume (ml)	Extraction time	Temperature (°C)	Fractions	Results of experiment in Figure/Table
vac	D	170	ether	500	24 hr	−196/20		1A
CO_2	D	170	ether	10	90 min	<20		1A
imm mw[b]	D	100	hexane	500	60 sec	(≫ ambient)		1A
dip	DT[e]	100	water	500	~2 sec	~96	water/ether	1B, 5B/2
dip	DT[e]	100	methanol	2 × 300	2 × 10 sec	(ambient)		1B/2
dip	DT[e]	100	methanol	2 × 300	2 × 30 sec	(ambient)	water/ether	1B, 5A/2
dip	DT[e]	200	CH_2Cl_2	2 × 600	2 × 30 sec	(< ambient)		1B/2
imm mw[c]	DT[e]	100	hexane	600	30 (~90) sec	(> ambient)		5A/2
imm mw[c]	DT[e]	100	hexane	600	60 (~120) sec	(≫ ambient)		1B/2
imm mw[d]	DD	100	hexane	600	60 (~120) sec	max. 52		4
imm mw[d]	H	100	hexane	600	60 (~120) sec	max. 47		4
imm mw[d]	DT[f]	100	hexane	600	60 (~120) sec	max. 53		2, 3
imm	DT[f]	50	hexane	300	120 sec	50–56		3
imm	DT[f]	50	hexane	300	120 sec	~22		3

[a]Abbreviations: vac = vacuum distillation; CO_2 = carbon dioxide extraction; dip = leaves dipped into solvent; imm = leaves entirely immersed in solvent; imm mw = leaves entirely immersed in solvent and heated in a microwave oven; D = *Daucus carota sativus*; DT = *Daucus carota sativus* cv. Tip-Top; DD = *Daucus carota sativus* cv. Danvers; H = *Heracleum sphondylium*.
[b]Microwave oven: Panasonic NN6452b run at 800 W.
[c]Microwave oven: Bauknecht MCCD 1820 Duo run at 900 W.
[d]Microwave oven: Panasonic NN-6807 run at 700 W.
[e]Sown 19.5.93, extracts prepared 63–80 days later.
[f]Sown 2.6.94, extracts prepared 115 days later.

Na_2SO_4 and filtered (Schleicher & Schuell No. 595½). Crude extracts and fractions were concentrated to 20 or 40 ml (1.25–10 gram leaf equivalents gle/ml) in a rotary evaporator at reduced pressure.

Volumes of 1.6 ml of the test solutions were applied with a glass chromatographic sprayer to surrogate leaves (hogweed shape; Figure 4 below) made of green paper and coated with a thin layer of paraffin (Merck, melting point 42–44°C) (Degen and Städler, 1997a). If not stated otherwise, 4 gle of extracts were sprayed onto each paper leaf model, which had an area of 100 cm^2. Hence, the concentration was only slightly above the average weight/area ratio of about 3.2 g/100 cm^2 for leaves of *Daucus carota sativus* cv. Tip-Top. For one oviposition bioassay (Figure 1A), we used plastic parsley leaves provided with a coat of paraffin wax.

Insects and Oviposition Choice Assays

Insects were obtained from a laboratory culture reared on carrots (Städler, 1971) for ≤13 generations after collection of pupae in the field at Wädenswil. Adult flies were fed a standard artificial diet containing sugar and yeast hydrolysate and were offered separately water and a 10% sugar solution. They were kept in a climate controlled room at 21 ± 1°C and 80 ± 5% relative humidity. Oviposition experiments were performed in cubic screen cages (0.34 m^3), in which flies continuously emerged from pupae, with the same illumination and photoperiod as for previous assays (Degen and Städler, 1997a).

Oviposition devices consisted of plastic dishes (diameter 9 cm, height 4 cm) with a wetted foam rubber lining covered with a black cloth and a black polyethylene grid (Städler, 1971). Surrogate leaves were kept upright by a metal wire, which protruded from the stem base and was attached with two rubber bands to the dishes. Real leaves were inserted through an opening in the center of the grid into a small plastic vial filled with water. An inverted black plastic pot with a 5- × 5-cm wide opening was placed over the dishes to reduce "stray" oviposition by females stimulated on other leaves (Degen and Städler, 1997b).

Eight oviposition dishes were spaced on the cage floor, midpoints about 20 cm distant from each other, around an apple seedling in the center (resting and copulation site). Depending on the number of treatments (two, four, or eight), the leaf models were assigned to the positions in a regular alternate sequence or in a random manner. An experimental period usually lasted one day, but occasionally up to three days. Positions were changed for each unit in such a way that each treatment was located once at each position. Between bioassays with surrogate leaves, flies were exposed to carrot leaves to avoid nondiscriminating behavior due to a build-up of eggs. The population in the cages usually consisted of 100–400 individuals (males and females).

Statistics

Results of egg counts were expressed as percentage per dish of total oviposition. We used the Friedman test and the Friedman test modified for repetitions of each treatment within a block (= one experimental period in a cage) to compare the results of egg counts. Multiple comparisons were made following the method (least significant rank sum difference) given by Conover (1980). The number of replicates (n) is given by the number of leaf models with identical treatment (m) multiplied by the number of experimental periods (b) during which they were tested (N = total number of eggs).

RESULTS

Microwave extracts with hexane as solvent elicited the strongest ovipositional response in both multiple choice experiments (Figure 1A and B). The vacuum distillate and the CO_2 extract did not stimulate more egg-laying than the

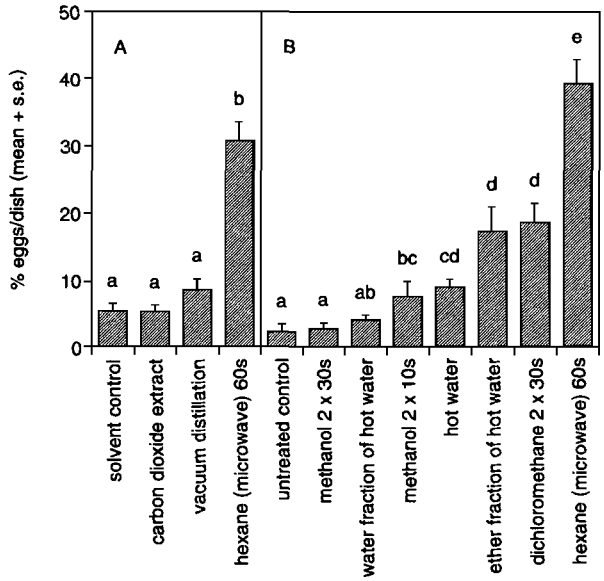

FIG. 1. Oviposition stimulating activity of different extracts of carrot leaves as measured in two multiple choice experiments (A and B). Friedman test for treatment effect: (A) $P < 0.001$; $m = 2$; $b = 6$; $n = 12$; $N = 3747$; (B) $P < 0.001$; $m = 2$, $b = 4$; $n = 8$; $N = 3273$. Each leaf model was sprayed with 5 g (A) or 4 g (B) leaf equivalents of the respective extracts. Columns topped by different letters differ significantly at $P < 0.05$.

solvent control. Leaf models treated with the ether fraction of the water extract or with the previously used dichloromethane washes received only half as many eggs as models treated with microwave extracts. The stimulatory activity of the extracts declined with increasing polarity of the solvent.

When we tested extracts separately against the solvent control in dual- or triple-choice experiments, we obtained essentially the same results as with the multiple comparison (Table 2). The extract/control ratios for oviposition recorded with both experimental approaches—dual/triple choice and multiple choice—were highly correlated (r = 0.98; P = 0.003; N = 5). The only major discrepancy was with the hot water extract: no net stimulatory effect could be detected in the dual-choice experiment, in contrast to the findings of the multiple-choice experiment. Stronger ovipositional responses occurred with microwave-assisted extractions lasting 60 sec than with extractions lasting only 30 sec. This effect is probably due both to the duration of the extraction and the higher temperatures achieved as a consequence of the prolonged extraction time. Extraction times could be prolonged further with a modified microwave oven allowing a reflux of the boiling hexane.

Carrot flies always laid significantly more eggs around leaf models treated

TABLE 2. OVIPOSITION BY CARROT FLIES BELOW SURROGATE LEAVES TREATED WITH EXTRACT AND SOLVENT: RESULTS OF DUAL- OR TRIPLE-CHOICE ASSAYS

Extract	Eggs/dish (%, mean ± SE)[a]	P (Friedman test)	Surrogates (m)	Experimental periods (b)	Total replicates (n)	Total eggs (N)
Solvent control	11.9 ± 1.3	NS	4	4	16	1156
Water	13.1 ± 1.2		4	4	16	
Solvent control	9.0 ± 1.0 a	<0.05	4	8	32	2308
Methanol 2 × 10 sec	19.4 ± 2.8 b		2	8	16	
Methanol 2 × 30 sec	12.6 ± 2.1 a		2	8	16	
Solvent control	3.7 ± 1.7	<0.005	4	2	8	730
Dichloromethane	21.3 ± 3.4		4	2	8	
Solvent control	1.9 ± 0.7 a	<0.001	4	4	16	1138
Hexane microwave 30 (~90) sec	16.5 ± 3.0 b		2	4	8	
Hexane microwave 60 (~120) sec	29.7 ± 3.6 c		2	4	8	

[a]Different letters indicate significant differences among the treatments at $P < 0.05$.

with microwave extract than around control leaves, even when the concentration of the extract was as low as 0.125 gle (Figure 2). For comparison, real carrot leaves of equal size weigh about 3 g. The fact that the 0.125 gle treatment elicited about four times more egg-laying than the solvent control suggests that measurable behavioral effects might be evoked with even lower concentrations. On the other hand, the dose–response curve reaches a plateau around 4 gle or may have an optimum in this region. The latter possibility cannot be ruled out, because concentrations above 8 gle were not included in the assays.

In the microwave oven, the water-containing plant tissues absorb radiation and transfer heat to the surrounding medium. A cold hexane extract was much less stimulating than the microwave extract (Figure 3). However, when preheated hexane was added to the leaves—the extraction parameters (temperature and duration) otherwise being comparable—the resulting extract was as active as the microwave extract (Figure 3). This implies that the solubility of the oviposition stimulants present in the leaf surface is enhanced by increasing the temperature of the solvent, irrespective of the heat source.

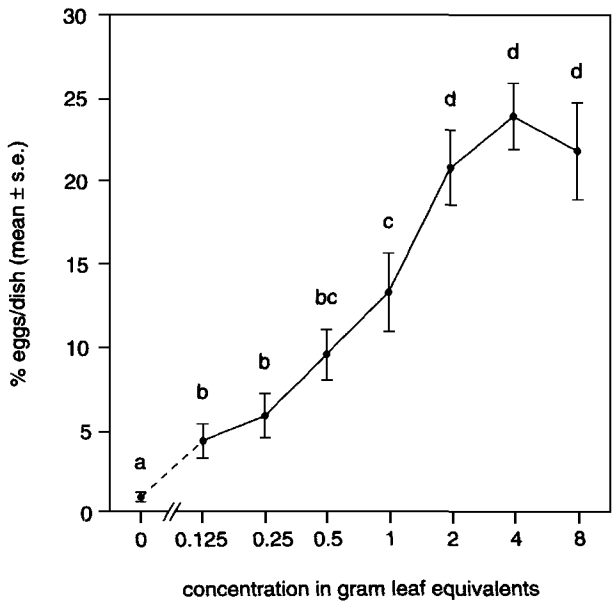

FIG. 2. Oviposition of carrot flies around surrogate leaves in response to increasing concentrations of microwave-assisted hexane extract of carrot foliage. Friedman test for treatment effect: $P < 0.001$; $m = 1$; $b = 8$; $n = 8$; $N = 4980$. Means accompanied by the same letter are not significantly different at the 5% level.

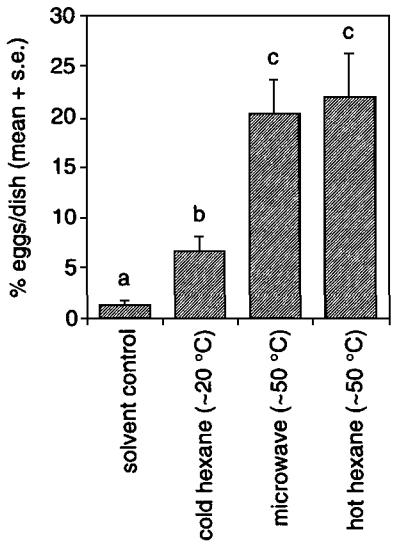

FIG. 3. Effect of solvent temperature during extraction on the stimulatory activity of three hexane extracts prepared from carrot foliage. Friedman test for treatment effect: $P < 0.001$; $m = 4$; $b = 2$; $n = 8$; $N = 2465$. Significant differences ($P < 0.05$) between the treatments are indicated by different letters.

Surrogate leaves sprayed with microwave extracts of carrot and hogweed plants at approximately natural concentrations (with respect to gle/leaf area) were only slightly less acceptable to carrot flies than real leaves (Figure 4). The difference between artificial and genuine leaves was only significant in the case of the carrot plant, and it was least pronounced on the first day of the experiment. This could be due to the fact that we supplied fresh leaf material for each experimental period, whereas we used the same leaf models throughout the four-day experiment. Evaporation of more volatile compounds or degradation may have led to a loss of stimulatory activity in the surrogate leaves.

The methanolic extract with the shorter extraction time slightly stimulated oviposition, whereas the one with the longer extraction time did not differ from the solvent control. Assuming that the latter contained at least the same quantity of stimulants as the former, we conclude that methanol extracted additional compounds that were deterrent, in increasing amounts with longer extraction times and possibly from further inside the leaf. We separated the "inactive" methanol extract into a stimulatory ether fraction and an inhibitory water fraction (Figure 5A). The stimulatory activity was not extractable from the methanol phase with hexane, which indicates that the active compounds must be of intermediate polarity. An analogous situation was found with the hot water extract

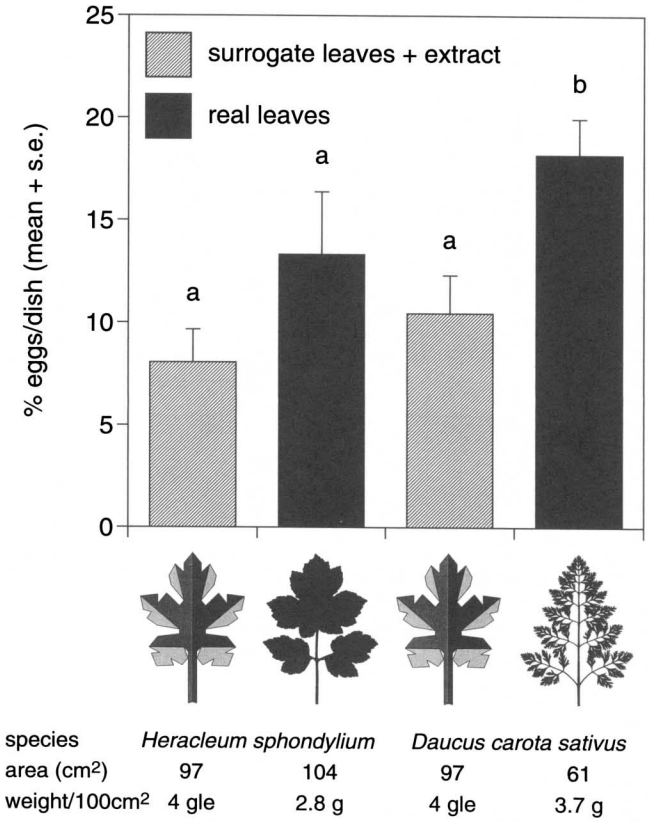

FIG. 4. Oviposition around surrogate leaves sprayed with microwave-assisted hexane extracts of carrot leaves, *Daucus carota sativus* cv. Tip-Top, and hogweed leaves, *Heracleum sphondylium*, compared to that around real leaves of the corresponding plant species (Friedman test for treatment effect: $P < 0.025$; $n = 8$; $N = 2879$). Significant differences ($P < 0.05$) among the treatments are indicated by different letters.

whose ether fraction showed stronger stimulatory activity than the crude extract (Figure 1B). The combined water and ether fraction elicited much less egg-laying than the ether fraction alone (Figure 5B).

DISCUSSION

A review of the extraction methods tested so far to isolate oviposition stimulants from carrot leaves shows that almost all extracts prepared from undamaged leaves stimulated oviposition to varying degrees (Table 3). In contrast, with

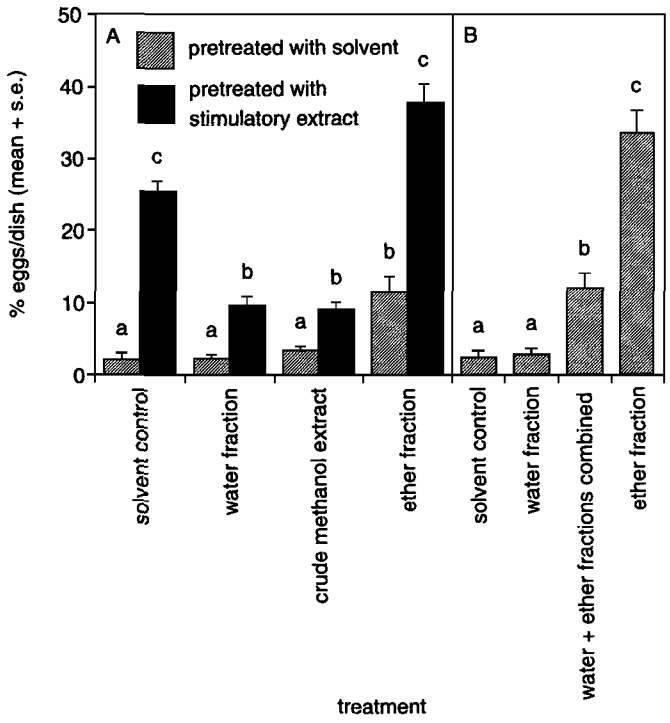

FIG. 5. Oviposition deterring activity of polar fraction of (A) methanol (2 × 30 sec; Friedman test for treatment effect: $P < 0.001$; $m = 2$; $b = 4$; $n = 8$; $N = 4321$) and (B) hot water extract ($P < 0.001$; $m = 2$; $b = 4$; $n = 8$; $N = 1735$) of carrot foliage. In experiment A, half the surrogate leaves were pretreated with a stimulatory microwave extract of carrot leaves. Different letters indicate significant differences between the treatments at $P < 0.05$.

the exception of the steam distillate (Berüter and Städler, 1971), none of the extracts of ground leaf material had an overall stimulatory effect, irrespective of the polarity of the solvent. The most plausible explanation for this phenomenon is a masking effect of inhibitory substances inside the leaf that are coextracted with the stimulatory compounds when the leaves are ground. Such inhibitory compounds previously were assumed to be present in a steam distillate of celery foliage (Städler, 1972). Here we confirm the occurrence of deterrents or—less likely—repellents in another host-plant species.

The microwave-assisted extraction procedure appears to yield optimal results in terms of stimulatory power, since the treated leaf models approached the stimulation achieved by real leaves. However, a high overall stimulatory

TABLE 3. STIMULATORY AND DETERRENT EFFECTS OF TOTAL AND SURFACE EXTRACTS OF CARROT LEAVES ON OVIPOSITION OF CARROT FLY[a]

Leaf parts	Method	Solvent	Temperature[b]	Fraction	Overall stimulatory activity	Stimulants	Deterrent activity	Reference
Total (leaves ground)	vacuum distillation	ether	−196/20	crude	−			a
	carbon dioxide	ether	11–19	crude	−			a
	steam distillation		120	petrol ether	+	pp[c]		c
	ground with solvent	ether	35 (bp)	crude	−			d
	ground with solvent	benzene	80 (bp)	crude	−			d
	ground with solvent	pentane	36 (bp)	crude	−			d
	ground with solvent	ethanol	ambient	water	−			d
				ether	−			d
				hexane	−			d
	('leaf juice')	(water)	ambient	crude	−			b
Surface (leaves cut, otherwise undamaged)	immersion	water with detergent[d] or complexing agent[e]	ambient	crude	±			e, f
	dippings	water	~96	crude	±			a
				water	−		+	a
				ether	+	fc[f]		a

dippings	methanol	~20	crude	±			a, f
			water	–		+	a
			ether	+			a
			hexane	–			a
dippings	dichloromethane	<ambient	crude	+			a
			ether[g]	+	pp, fc, pa		e, g
immersion	petrol ether	ambient	crude	+			d
immersion	hexane	~20	crude	+			a
immersion	hexane	~50	crude	++			a
microwave	hexane	~50	crude	++		(–)	a
(immersion)			ether[g]	++	pp, fc, pa		h
			methanol[g]	+			h
dippings	melted paraffin	70	crude	+			d

[a]Abbreviations: effect on oviposition: – = no activity, ± = week activity, + = moderate activity, ++ = strong activity; stimulants: pp = phenylpropenes, fc = furanocoumarins, pa = polyacetylenes; references: a = this study, b = Bohlen (1967), c = Berüter and Städler (1971), d = Städler (1972), e = Städler and Buser (1984), f = Städler (1986), g = Städler et al. (1990), h = Degen and Städler (1998).

[b]Temperature during rotary evaporation may be higher, e.g., for aqueous solutions up to 70°C.

[c]*trans*-Methylisoeugenol.

[d]1% Tween 80 or 1% Triton X-100.

[e]1‰ β-cyclodextrin.

[f]Good method for extraction of furanocoumarins (Zobel and Brown, 1988), yet carrot plants usually contain only low concentrations of furanocoumarins (Städler and Buser, 1984; Ceska et al., 1986; Städler et al., 1990) that may not account for the comparatively high stimulatory activity of the ether fraction.

[g]Fractionation on silicagel column (Städler et al., 1990).

activity is not the only criterion for a suitable extraction method. In studies of host-plant selection, the absolute yield of active compounds is of secondary importance. In general, surface extracts may prove to be more suitable for comparative purposes, since they should be more representative than total extracts of the compounds that can actually be perceived by the insects (Städler and Roessingh, 1991). The stimulatory activity of microwave extracts of various host plants explained a fair amount of the variation observed in acceptability of these plant species to egg-laying carrot flies (Degen and Städler, 1998). The results of this study for hogweed and carrot support this finding, although the differences were significant only between real leaves and not between microwave extracts.

An ideal extraction method should selectively target active compounds present in the uppermost wax layers while avoiding leakage of substances from the leaf interior as much as possible. Clearly some solvents do not meet such a requirement. For example, the exposure of leaves to methanol will inevitably lead to the extraction of polar compounds from inside the cells unless the duration of solvent contact is very short. The brief dipping of leaves into almost boiling water removes substantial amounts of the cuticular wax, including compounds that are associated with deeper layers of the cuticle. Bioassays, however, have failed to reveal any significant leakage from inside the leaf due to cell disruption, as the epidermal cells seem to remain alive (Zobel and Brown, 1988). Similarly, Städler and Buser (1984) claimed that dichloromethane washings do not disrupt epidermal cells and, therefore, the resulting crude extracts contain little material from the leaf interior. In contrast, the sudden rise in temperature inside plant material that accompanies microwave-assisted extraction causes severe damage to cells (Paré et al., 1991). However, it is questionable whether substances of cytoplasmic or vacuolar origin, which are released, penetrate the cuticle and are taken up by the apolar extraction medium in substantial amounts. The hot hexane extracts without microwave irradiation were similar in appearance (almost uncolored) and stimulated oviposition as effectively as microwave extracts, although they most probably were much less detrimental to leaf cells. Even if hot hexane also extracts fat-soluble compounds from inside the leaf (e.g., from oil ducts, intercellular space), the bulk of extracted substances may still originate from the leaf surface. In any case, there is no experimental evidence or theoretical reason supporting the assumption frequently made that chloroform or any other apolar solvent does not enter the cuticle during short dippings or rinsings (Riederer and Schneider, 1989). Such penetration is not a serious problem as long as the distribution of secondary metabolites is fairly consistent throughout the apoplast and the cuticle. According to Derridj et al. (1996), the chemical information available to insects on the leaf surface may be similar to the apoplastic composition.

The yield of soluble cuticular lipids (alkanes, alkanols, acids, esters) varies not only quantitatively, but also qualitatively, with extraction methods, e.g., dipping versus rinsing (Stammiti et al., 1996), solvent type, duration of immer-

sion, and temperature (Riederer and Schneider, 1989). It seems plausible that this holds true also for secondary compounds embedded in the matrix built up by cuticular lipids. Since we did not analyze our extracts for known oviposition stimulants, we do not know to what degree variation in stimulatory power among different extracts is explained by the quantities extracted. Semiochemicals previously identified in dichloromethane extracts undoubtedly contribute to the activity of extracts using other solvents, e.g., hexane (Degen et al., 1999). The results suggest that host choice of the carrot fly is also affected by polar inhibitors as well as by unidentified stimulatory compounds. This implies that no single extraction method is optimal for all key compounds mediating oviposition preferences. For a complete picture, it may be necessary to make comparative extractions of a range of host plants, because different host species may contain distinct sets of stimulants and deterrents with differing solubilities (Städler et al., 1990; Degen and Städler, 1998).

Since oviposition stimulants for different insects differ in polarity and hence solubility, and since even insects that share the same host plants do not necessarily react to the same compounds (Städler, 1994), no prediction of extraction efficiency can be made (Städler and Roessingh, 1991). This study highlights the need to look at various methods. In particular, when the sampling behavior of the insect (e.g., before oviposition) does not involve damaging the leaf, surface extracts from intact plant material should be the method of choice. Surface extracts are easy to produce and fewer steps are required for purification because fewer compounds are present. Hot hexane extracts may be suitable in cases where the essential semiochemicals are of low or intermediate polarity. The microwave technique also is applicable with other, partially absorbing solvents such as methanol or dichloromethane (Paré et al., 1991) and offers a rapid extraction method for bulk production without the need to heat large volumes of solvents.

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IDENTIFICATION OF FEMALE SEX PHEROMONE OF THE LEGUME POD BORER, *Maruca vitrata* AND ANTAGONISTIC EFFECTS OF GEOMETRICAL ISOMERS

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Abstract—An EAD-active component in a female abdominal tip extract of the legume pod borer, *Maruca vitrata* (Fabricius) (= *M. testulalis*), was identified as (*E*,*E*)-10,12-hexadecadienal (*E*10,*E*12–16 : Ald). In laboratory bioassays, the purified synthetic *E*10,*E*12–16 : Ald with 99% isomeric purity had attractancy to male moths equal to the crude extract. However, the unpurified chemical with 92% isomeric purity did not show any attractancy. Addition of 0.1 ng *EZ* isomer or 1 ng of *EZ*, *ZE*, or *ZZ* isomers of 10,12–16 : Ald to 1 ng of the purified synthetic pheromone antagonized its attractancy as did addition of each isomer to 1 female equivalent of the crude extract. These results suggest that it is necessary to develop formulation and release techniques that maintain extremely high isomeric purity of the synthetic pheromone for male trapping in the field.

Key Words—Sex pheromone, legume pod borer, *Maruca vitrata*, Pyralidae, Lepidoptera (*E*,*E*)-10,12-hexadecadienal, attraction, geometrical isomer, isomeric purity, antagonistic effect.

INTRODUCTION

The legume pod borer, *Maruca vitrata* (Fabricius) (= *M. testulalis*), is a serious insect pest of leguminous crops including cowpea, pigeon pea, and common bean in the tropics and subtropics of Africa, Asia, Latin America, and Oceania. The grain yield reduction by this pest is estimated to be 20–60% (Singh and Emden, 1979), and an integrated pest management method urgently

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needs to be established. The biology of the pest has been detailed in several reports and reviews (Taylor, 1978; Singh et al., 1990; Jackai and Singh, 1991), but there have been no studies on mating behavior and sex pheromone except for a few preliminary trials (Okeyo-Owuor and Agwaro, 1982; Jackai et al., 1990).

Witzgall et al. (1993) and McDonough et al. (1996) described antagonistic effects of impurities on attractancy of synthetic pheromone in Lepidoptera. Geometrical isomers of the major pheromone component are particularly important because they frequently contaminate the pheromone through the synthetic process. In some cases, an isomer or decomposition product can be introduced into a pure synthetic pheromone preparation by environmental conditions. If such isomers antagonize the pheromonal activity, they may reduce the effectiveness of pest management.

In this paper, we describe the identification of the female sex pheromone of the legume pod borer and the antagonistic effects of the geometric isomers of the synthetic pheromone on its attractancy.

METHODS AND MATERIALS

Insects. The pest larvae were collected from the cowpea field at the Crops Research Institute, Fumesua, Ghana, and imported to Japan by air. In our laboratory, larvae were reared on artificial diets, Silkmate L-3 (S) (for the first and second instars) and Insecta LF (the third to fifth instars) purchased from Nihon-Nôsan-Kôgyô, Yokohama, Japan. The insects were reared under a 13L : 11D photoperiod at 22–24°C and 70–80% relative humidity. Pupae were sexed and separately placed in a stainless-steel-screen cage (30 × 30 × 30 cm). After emergence, both sexes were kept under the same conditions and provided with a 5% sucrose solution as food from a paper towel in the cage.

Extraction. Abdominal tips, containing pheromone glands, were excised from 6- to 7-day-old adult female moths after 9–10 hr of lights off, then extracted with hexane (1 μl/tip) for 10 min. The extract was pooled to a few hundred female equivalents in a conical glass vial, which was sealed with hexane in a larger glass vial to prevent evaporation and stored at −20°C.

Gas Chromatography. For comparing retention times of an EAD-active component and synthetic chemicals, gas chromatography (GC) was conducted on a Shimadzu GC-14B equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas at a column head pressure of 120 kPa. Two fused silica capillary columns with different polarity—a nonpolar DB-1 and a medium-polar DB-Wax (30 m × 0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, California)—were used for analyses. Injections were made in the splitless mode at an initial column temperature of 80°C. After 2 min, the

injector was changed to the split mode and the column temperature increased from 80 to 220°C at 7°C/min.

GC-EAD. A crude female tip extract and synthetic pheromone components were analyzed by a gas chromatograph equipped with an electroantennographic detector (GC-EAD). It was set up with a Hewlett-Packard (HP) 5898 and a fused silica capillary column (HP-Innowax, 30 m × 0.25 mm ID) according to Struble and Arn (1984) with some modification.

GC-MS. Gas chromatography–mass spectrometry (GC-MS) was conducted with a Shimadzu QP1000EX coupled with a Shimadzu GC-14A GC and a Sanyo data analysis system. A DB-23 fused silica capillary column (30 m × 0.25 mm ID, 0.25 μm, J&W Scientific) was used under the same analytical conditions mentioned above for the GC-14B, but helium was used as the carrier gas. For electron impact (EI) mass spectrometry, the ionization voltage was 70 eV and temperature of the ion source and interface were 250°C and 270°C, respectively. Chemical ionization (CI) mass spectrometry was conducted with isobutane as reagent gas. Ionization voltage, ion source temperature, and interface temperature were 200 eV, 100°C, and 200°C, respectively.

Chemicals. Synthetic (*E*,*E*)-10,12-hexadecadienal with 92% isomeric purity was obtained from the Laboratory of Bioregulation Chemistry, Tokyo University of Agriculture and Technology. It was used for bioassays without purification. *EE*, *EZ*, *ZE*, and *ZZ* isomers of 10,12-hexadecadienal were provided as a mixture (61% *EE*, 19% *EZ*, 16% *ZE*, 5% *ZZ*) from Shin-Etsu Chemical Co., Ltd., Tokyo, Japan. The mixture was separated and purified on a GC-14B gas chromatograph equipped with a wide-bore fused silica column, DB-Wax Megabore (30 m × 0.53 mm ID, 1 μm film thickness, J&W Scientific). The column temperature, initially 100°C for 2 min, was programmed to increase to 200°C at 5°C/min and thereafter held at the final temperature. After passing through the retention time measured in advance for each isomer, column exhaust was introduced into a glass tube (30 cm × 2 mm ID) for ca. 0.2 min. Then the inside surface of the tube was rinsed with 50 μl of hexane, and the rinse was collected into a conical glass vial. In this way, *EE*, *EZ*, *ZE*, and *ZZ* isomers were separated with isomeric purities of no less than 99%, 97%, 93%, and 81%, respectively.

Bioassays. Bioassays for both pheromone identification and antagonism effects of geometric isomers were conducted in wood-framed-screen cages (30 × 20 × 30 cm) set up in a dark room (5 m^2) equipped with a ventilator. A small electric fan was set beside the cage so that the wind blew in parallel with the longer axis of the cage. Ten male moths aged more than 7 days were released in a cage under the same conditions as used for insect rearing. The assays were conducted 6–9 hr after lights off. An individual assay was started by suspending a fan-shaped filter paper strip (45 mm × 45°) that was impregnated with a hexane solution of test material(s) from the top screen in the cage. Each assay lasted 5

min. Male response to the materials was judged by comparing attraction indices between treated and control (solvent alone) filter papers. The indices recorded for pheromonal identification were as follows: Orientation flight (OF) was the total number of flying males oriented to the filter paper with antennae extended forward and exposed hairpencils from the lower part of the abdomen, counted at 1-min intervals from the start of the assay, maximum orientation (MO) was the maximum number of males taking an orientation flight during assays, and source contact (SC) was the total number of males in contact with the baited filter paper for 5 min. In the assays for antagonism effects of geometrical isomers, filter paper strips were treated with 0.01, 0.1, or 1 ng of each isomer added to 1 ng of synthetic pheromone component or 1 female equivalent (FE) of crude abdominal tip extract. The bioassay was conducted as described above. Values of SC were compared between each treatment and the control, which was treated with a synthetic pheromone or crude extract alone.

RESULTS

Identification of an EAD-Active Component. A typical gas chromatogram of crude female abdominal tip extract is shown in Figure 1, exhibiting 16 peaks

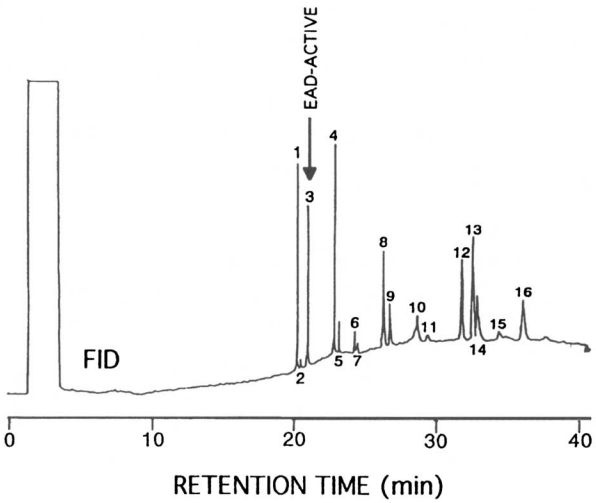

FIG. 1. Flame ionization detector (FID) response to female abdominal tip extract of *M. vitrata*. Only one peak (peak 3) coincided with that detected by the electroantennographic detector (EAD). GC: Shimadzu GC-14B, 2 min splitless injection; injector and FID temperature: 250°C; column: DB-Wax (30 m × 0.25 mm ID); temperature program: 80°C (2 min), 7°C/min to 220°C.

detected with the FID. Among those peaks, only one (peak 3) coincided with an EAD-active peak in retention time. EI and CI mass spectra of the EAD-active component(s) that appeared as peak 3 are shown in Figures 2A and 3A, respectively. In the EI mass spectrum, a fragment ion of m/z 67 displayed maximum intensity, and ions of m/z 81, 95, 109, and 236 $[M]^+$ also were obtained. Compared with EI mass spectra reported by Ando et al. (1988), the spectrum of the component in peak 3 was very similar to those of conjugated hexadecadienal with a molecular weight of 236. In the CI mass spectrum, ions of m/z 183 $[b]^+$ and 237 $[M + 1]^+$ were most abundant, followed by those of m/z 184, 223, 86, 293 $[M + 57]^+$, 85 $[a]^+$, 209, and 275 $[M + 39]^+$. Doolittle et al. (1985), in a case study of conjugated dienes by CI mass spectrometry, reported characteristic fragmentation depending on the double-bond position. According to them, the presence of the diagnostic ions, m/z 85 $[a]^+$ and 183 $[b]^+$ indicated that the double-bond position was at 10 and 12 in the hydrocarbon chain of hexadecadienal (see the scheme in Figure 3). GC-EAD analysis of four synthetic geometrical isomers of 10,12-hexadecadienal revealed all to have EAD activity. Retention time of the *EE* isomer was identical to that of peak 3 on both DB-1 and DB-Wax columns (Table 1). Furthermore, both EI and CI mass spectra of

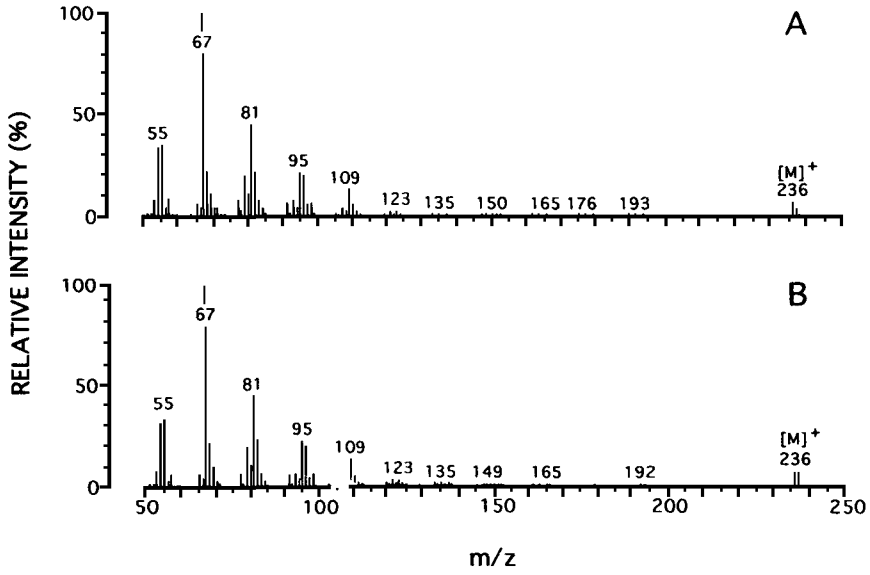

FIG. 2. EI mass spectra of the EAD-active component in peak 3 (A) and synthetic (*E*,*E*)-10,12-hexadecadienal (B). MS: Shimadzu QP1000EX; ionization voltage: 70 eV; ion source temperature: 250°C; interface temperature: 270°C.

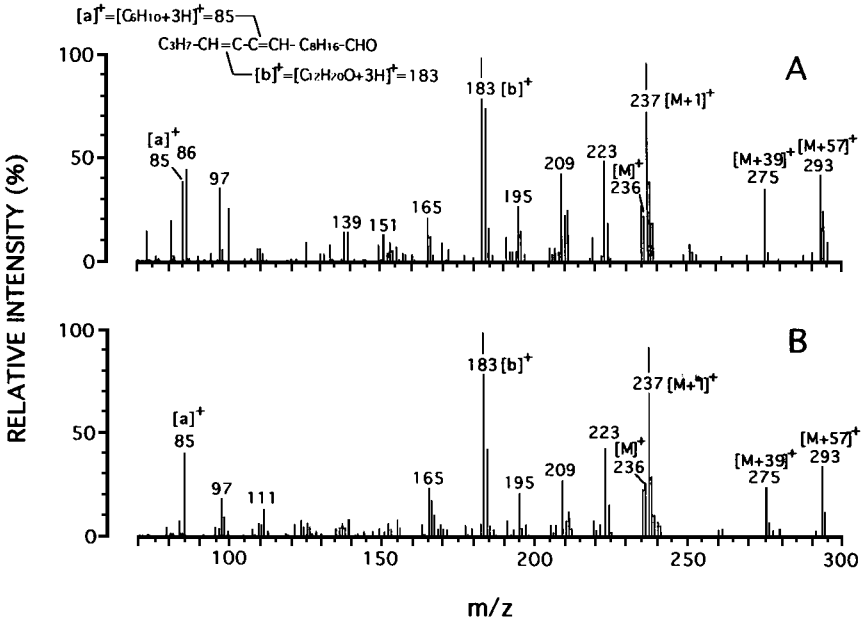

FIG. 3. CI mass spectra of the EAD-active component in peak 3 (A) and synthetic (*E*,*E*)-10,12-hexadecadienal (B). The scheme above the spectra represents the fragmentation of diagnostic ions of $[a]^+$ and $[b]^+$, modified from Doolittle et al. (1985). MS: Shimadzu QP1000EX; reagent gas: isobutane; ionization voltage: 200 eV; ion source temperature: 100°C; interface temperature: 200°C.

TABLE 1. GC RETENTION TIMES OF FOUR GEOMETRICAL ISOMERS OF SYNTHETIC 10,12-HEXADECADIENAL AND PEAK 3 ON TWO DIFFERENT CAPILLARY COLUMNS[a]

Isomer	Retention time on columns (min)	
	DB-1	DB-Wax
ZE	19.06	20.37
EZ	19.18	20.57
ZZ	19.35	20.74
EE	19.46	20.89
Peak 3	19.46	20.89

[a]GC: Shimadzu GC-14B, 2 min splitless injection; injector and FID temperature: 250°C; column: DB-1 and DB-Wax (30 m × 0.25 mm ID); temperature program: 80°C (2 min), 7°C/min to 220°C.

the synthetic *EE* isomer were identical to those of the component in the extract (Figures 2B and 3B). Therefore, the EAD-active component in the female tip extract was identified as (*E*,*E*)-10,12-hexadecadienal (*E*10,*E*12–16 : Ald). The average amount of the component in crude extract was quantified from a peak area on the gas chromatogram as 0.9 ± 0.6 (SD) ng/FE.

Bioassays of Crude Extract and Synthetic Component. Male responses to purified or unpurified synthetic *E*10,*E*12–16 : Ald and crude extract are summarized in Table 2. No male response to unpurified *E*10,*E*12–16 : Ald was observed irrespective of the amount, but the chemical purified to 99% attracted male moths. In each behavioral category, statistically the level of attraction of purified chemical was generally as high as that of crude extract. Although generally there were large variances in values, attractancy tended to increase with the amount of compounds when crude extract and purified chemical were tested.

Antagonistic Effects of Geometrical Isomers. The addition of 0.1 ng *EZ* isomer or 1 ng of *EZ*, *ZE*, or *ZZ* isomers caused a significant antagonism effect on the attractancy of synthetic pheromone (Table 3). Moreover, when 0.1 ng *EZ* isomer or 1 ng of *EZ*, *ZE*, or *ZZ* isomers was added to crude female abdominal tip extract, attractancy to male moths was reduced (Table 4).

TABLE 2. RESPONSES OF MALE *M. vitrata* TO PURIFIED AND UNPURIFIED (*E*,*E*)-10,12-HEXADECADIENAL AND CRUDE FEMALE ABDOMINAL TIP EXTRACT

Treatment	Amount[a]	Attraction index (Mean ± SD)[b]		
		OF	MO	SC
Crude extract	10 FE	10.7 ± 5.7 a	14.8 ± 5.9 a	24.3 ± 14.9 a
	1 FE	7.0 ± 5.7 a	4.1 ± 2.0 b	22.4 ± 15.1 a
(*E*,*E*)-10,12–16:Ald purified[c]	10 ng	5.8 ± 1.8 ab	4.7 ± 0.5 b	12.7 ± 2.3 ab
	1 ng	5.9 ± 3.0 a	3.3 ± 0.8 bc	8.7 ± 5.7 b
(*E*,*E*)-10,12–16:Ald unpurified[d]	10 ng	0.3 ± 0.7 b	0.3 ± 0.5 c	2.1 ± 1.6 b
	1 ng	0.4 ± 0.7 b	0.4 ± 0.5 c	1.8 ± 1.2 b
Control[e]		1.0 ± 0.4 b	0.3 ± 0.5 c	1.0 ± 1.3 b

[a] Female equivalent (FE) of crude abdominal tip extract or nanograms (ng) of synthetic chemical treated to filter paper in hexane solvent.

[b] Means in the same column followed by the same letter are not significantly different at $P < 0.05$ by the Tukey-Kramer's test. OF = orientation flight, MO = maximum orientation, SC = source contact; see text for details.

[c] 99% isomeric purity.

[d] 92% isomeric purity.

[e] Filter paper treated with solvent alone.

TABLE 3. ANTAGONISTIC EFFECTS OF *EZ*, *ZE*, AND *ZZ* ISOMERS OF 10,12-HEXADECADIENAL ON MALE *M. vitrata* ATTRACTION TO (*E*,*E*)-10,12-HEXADECADIENAL

Treatment[a]	SC (mean ± SD)[b]	Antagonism index (%)[c]	*P*[d]
1 ng *EE* + 1 ng *EZ*	0.7 ± 1.0	92	<0.01
1 ng *EE* + 1 ng *ZE*	1.0 ± 0.7	89	<0.05
1 ng *EE* + 1 ng *ZZ*	1.4 ± 1.7	84	<0.05
1 ng *EE* + 0.1 ng *EZ*	2.6 ± 1.8	70	<0.05
1 ng *EE* + 0.1 ng *ZE*	5.6 ± 5.0	36	0.3246
1 ng *EE* + 0.1 ng *ZZ*	6.8 ± 6.5	22	0.5726
1 ng *EE* + 0.01 ng *EZ*	6.8 ± 4.0	22	0.5218
1 ng *EE* + 0.01 ng *ZE*	6.0 ± 2.9	31	0.3957
1 ng *EE* + 0.01 ng *ZZ*	10.5 ± 5.2	−21	0.5972
1 ng *EE* (control)	8.7 ± 5.7		

[a] Each amount of *EZ*, *ZE*, or *ZZ* isomers of 10,12-hexadecadienal was treated with 1 ng of *EE* isomer to filter paper in hexane.
[b] Source contact; see text for details.
[c] Index of antagonism (%) = 100 [(Cc − Ct)/Cc], Cc = SC in control, Ct = Sc in each treatment.
[d] Unpaired-sample *t* test between SC in the treatment and control.

TABLE 4. ANTAGONISTIC EFFECTS OF *EZ*, *ZE*, AND *ZZ* ISOMERS OF 10,12-HEXADECADIENAL ON MALE *M. vitrata* ATTRACTION TO CRUDE FEMALE ABDOMINAL TIP EXTRACT

Treatment[a]	SC (mean ± SD)[b]	Antagonism index (%)[b]	*P*[b]
1 FE EXT + 1 ng *EZ*	0	100	<0.01
1 FE EXT + 1 ng *ZE*	0.2 ± 0.4	99	<0.01
1 FE EXT + 1 ng *ZZ*	0.7 ± 0.8	97	<0.01
1 FE EXT + 0.1 ng *EZ*	4.2 ± 2.8	81	<0.05
1 FE EXT + 0.1 ng *ZE*	9.8 ± 4.3	56	0.0951
1 FE EXT + 0.1 ng *ZZ*	7.8 ± 3.4	65	0.0558
1 FE EXT + 0.01 ng *EZ*	20.2 ± 9.4	10	0.7723
1 FE EXT + 0.01 ng *ZE*	22.6 ± 9.0	−1	0.9788
1 FE EXT + 0.01 ng *ZZ*	33.3 ± 16.6	−49	0.1976
1 FE EXT (control)	22.4 ± 15.1		

[a] Each amount of *EZ*, *ZE*, or *ZZ* isomers of 10,12-hexadecadienal was treated with 1 female equivalent (FE) of crude abdominal tip extract (EXT) to filter paper in hexane.
[b] See footnotes [b–d] of Table 3.

DISCUSSION

It is clear that *E*10,*E*12–16 : Ald is a sex pheromone itself or, at least, a major component of the female sex pheromone of the moth. The possibility that a minor component(s) exists in the extract can not be excluded because the attractancy of the synthetic chemical was generally lower than that of the crude extract. Based on pheromonal identifications in Lepidoptera, multicomponent pheromone systems probably are important for reproductive isolation of sympatric species, and many moths have them (Tamaki, 1985). Improvements in analytical sensitivity of GC and/or GC-MS may lead to the detection of minor components from crude extracts in this moth as well. We detected the corresponding alcohol, *E*10,*E*12–16 : OH at 3–4% of the pheromonal aldehyde. However, the alcohol did not increase behavioral activity when added to the aldehyde.

In contrast to the purified synthetic chemical, no attractancy was shown by the unpurified synthetic chemical. The failure of attraction was likely caused by the geometrical isomers of *E*10,*E*12–16 : Ald. This is supported by the findings that addition of *EZ*, *ZE*, or *ZZ* isomers to lures containing the synthetic pheromone and the crude extract antagonized their attractancy to male moths. Among the three isomers, *EZ* isomer was most effective as an antagonist with 10% addition to the lures having an effect. Several studies have suggested the importance of geometrical isomers of pheromones for practical use in pest control. (*E*, *E*)-8,10-Dodecadien-1-yl acetate (*E*8,*E*10–12 : Ac) has been identified as the female sex pheromone of the pea moth, *Cydia nigricana* (Greenway, 1984). Attraction of male moths to synthetic *E*8,*E*10–12 : Ac declines rapidly after only a few days in the field (Greenway and Wall, 1981; Wall et al., 1987). In the laboratory, Witzgall et al. (1993) showed that 4% of *EZ*, *ZE*, and *ZZ* isomers were detected from the rubber septa after eight days of a purified *E*8,*E*10–12 : Ac application. Furthermore, the addition of any one of the isomers (5% final concentration) to fresh lures of *E*8,*E*10–12 : Ac significantly reduced male attraction.

In general, conjugated double bonds are isomerized to an equilibrium mixture of four possible geometrical isomers when exposed to high temperatures and/or sunlight, atmospheric oxygen, and humid conditions (Ideses and Shani, 1987). This present study showed that extremely high isomeric purity of the pheromone component is necessary for male attraction in *M. vitrata*, and it may be difficult to use the synthetic pheromone to trap males in the field because of the inherent instability of aldehydes under field conditions. To solve this problem, two methods are proposed, i.e., mating disruption with mixtures of antagonists and use of pheromone derivatives. An equilibrium mixture of codlemone, (*E*, *E*)-8,10-dodecadien-1-ol, and its isomers disrupted mating of the codling moth, *Cydia pomonella*, to a larger extent than pure codlemone (McDonough et al., 1996). An example of the latter is propheromone, which is synthesized as

a metal complex of geometrical isomers including the pheromone component. When loaded in a dispenser, the propheromone slowly decomposes to pheromone on exposure to ambient air. This reaction is highly stereoselective and only isomers with a specific configuration are released (Streinz et al., 1993). Streinz et al. (1993) baited traps with the propheromone of *C. nigricana*, *E*8, *E*10–12 : Ac, and obtained good results in trapping male moths in the field.

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PREDICTING ATMOSPHERIC CONCENTRATION OF PHEROMONE IN TREATED APPLE ORCHARDS

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Abstract—A Lagrangian model was developed to predict the vertical distribution of pheromone in apple orchards treated with synthetic pheromone released from polyethylene tubing dispensers. Measurements of tree dimensions' dispenser heights, air temperature, and wind speed were used as inputs to the model. Data to test the model output were obtained by air sampling and capillary gas chromatography to determine atmospheric pheromone concentration. The model predicted highest concentrations of pheromone in the plane of the dispensers. Predicted and measured concentrations were in the range 0.5–5 ng/m^3 for blocks treated with 1000 or 2000 dispensers/ha. Mean wind speed had a large influence on pheromone concentrations within the canopy with concentrations decreasing at higher wind speeds. Wind speeds <0.1 m/sec, which represent good flying conditions for moths, resulted in high levels of mean pheromone concentration. Dispenser height had only a small influence on the maximum pheromone concentration, with the peak concentrations decreasing with increasing application height. The lower peak concentration for an elevated dispenser occurred mainly because wind speeds were higher in the upper parts of the tree canopy. Air temperature, dispenser density, and pheromone release rate (as inferred by dispenser liquid length), also had a significant influence on pheromone concentration because of the linear relationship between these parameters and the corresponding flux of pheromone released into the treated orchards. We use known scaling relationships to demonstrate these effects.

Key Words—Pheromone, mating disruption, Lagrangian model, atmospheric concentration, turbulence.

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INTRODUCTION

The most widely used dispenser system for mating disruption of insects in fruit crops is based on polyethylene tubing dispensers (Shin-Etsu Fine Chemical Co., Tokyo). These dispensers are formulated with a blend of pheromone compounds to offer protection against more than 20 pest species found in a range of crops worldwide. Successful mating disruption relies on the exposure of a male insect to an amount of pheromone sufficient to mediate his mating behavior. It is well documented that the maintenance of a sufficient concentration of pheromone over a certain time interval will prevent mate location (Cardé and Minks, 1995). However, many practical questions remain regarding the best deployment of these dispensers in order to optimize mating disruption.

One of the keys to improving the success of the disruption method is to improve our understanding of the impact of key variables on atmospheric concentrations of pheromone, which show rapid and large-scale fluctuations caused by the dispensers (Karg and Suckling, 1997). These variables include environmental factors (e.g., wind speed, turbulence, air temperature, etc.) and operational factors (e.g., dispenser type and release rate, distribution, height in the crop). There are many technical difficulties in obtaining an absolute measurement of concentration because the concentrations of pheromone in treated crops are typically low, of the order of only a few nanograms per cubic meter, requiring extended sampling intervals of several hours (Caro et al., 1980). Potentially valuable information on the rapid changes in pheromone concentrations is lost with chemical analysis after air sampling.

Previous studies of atmospheric pheromone concentrations (Caro et al., 1980; Flint et al., 1990) have been made by using higher than normal quantities of pheromone. Such studies have reported only anecdotal measurements (Witzgall et al., 1996), without investigating the impact of the different operational and environmental variables noted above. Furthermore, most studies of pheromone efficacy only report release rates from dispensers and fail to relate these to an atmospheric concentration (Lawson et al., 1996). Thus, there are still many gaps in our knowledge of how atmospheric concentrations of pheromone are influenced by the local environment within treated orchards. These gaps must be filled if we are to improve our understanding of mating disruption as a pest management tactic. One way is to develop appropriate models to describe how pheromone is released from the dispensers (Bradley et al., 1995) and subsequently transported through the canopy air space.

Wind turbulence is the main mechanism responsible for the atmospheric transport of scalars, such as heat, water vapor, CO_2, aerosols, and pheromone molecules. It is possible to predict the distribution of these scalars in an orchard environment, providing we can predict the turbulent properties of the wind regime and providing we can also describe the corresponding sources or sinks of

these scalars. In treated orchards, the scalar source density is easily determined by the product of the release rate per dispenser multiplied by the number of dispensers per unit ground area. However, we are still searching for a physically sound description of how the scalars are transported in plant canopies. The search currently follows two lines of inquiry, namely, a Eulerian model, in which both the wind field and the corresponding concentration are calculated at a fixed point in space, and a Lagrangian model (Sawford, 1985), in which just the concentrations are calculated by following the motions of many "marked fluid particles" that move with the turbulent flow.

In this paper, we consider a Lagrangian model based on wind statistics that are known a priori. The theory is simpler than the Eulerian counterpart, since it is concerned only with the dispersion or turbulent mixing of a scalar as it is released into a known wind flow. The Lagrangian model also takes more appropriate account of the effects of fluid particle history by following the likely path of a marked fluid particle from its time of release and by using probability functions that are based on the statistical properties of the turbulent wind. Tracking particle motion is important for modeling scalar transport within the canopy because the turbulent motions that are carrying the pheromone-laden air remain coherent over finite time and length scales. It is these large, coherent motions that are credited with the failure of Eulerian models within plant canopies, and the subsequent promotion of Lagrangian models as an appropriate alternative (Raupach, 1987).

Within a plant canopy, the dominant turbulent eddies are of the scale of the canopy height, and hence their motion persists through much of the canopy depth. Typically, the fluid motions exhibit a "persistence" over time intervals that are the order of the Lagrangian time scale, t_L. This time scale depends on the mean flow and the height of the plant canopy, as we define later (equation 12). Raupach (1987, 1989) developed the concept that the concentration field at any point in the canopy reflects the superposition of both a near-field and the far-field turbulent mixing process. This is because the scalar material arriving at any observation point has originated from both nearby and distant source points. Raupach (1987, 1989) included this near-field/far-field concept into his Lagrangian model of scalar concentration in plant canopies. Scalar dispersion is assumed to be nondiffusive in the near-field, where the travel time is small compared to t_L, i.e., in the near-field, close to a source point, the particle trajectories depart little from straight lines and so a pheromone cloud grows linearly with time. On the other hand, scalar dispersion is diffusive in the far-field, where the travel time is large compared to t_L. Persistence on the time scale of t_L has a negligible effect, and so the particle trajectories behave as a random walk, just as for a diffusion process. In this case, the pheromone cloud grows as the square-root of time.

Here, we have chosen a practical Lagrangian model (Raupach, 1989), to investigate its potential for predicting mean pheromone concentrations in treated orchards. With the help of this model, we hope to determine the optimal location

for pheromone dispensers under different environmental conditions by examining the influence of dispenser placement on mean pheromone concentration. The optimum dispenser placement will, in all likelihood, vary for different environmental conditions and for different orchard layouts. Optimizing the application parameters for dispensers offers the potential to increase the efficacy of mating disruption and to improve the cost–benefit ratio of mating disruption.

In this paper, we present a Lagrangian model for the transport of pheromone in treated orchards, and compare some predictions with measurements of atmospheric concentrations of pheromone estimated by air sampling. We also demonstrate the use of the model, by investigating the impact of wind speed, dispenser height, dispenser age, and air temperature on atmospheric pheromone concentrations.

METHODS AND MATERIALS

Theory. We propose a simple model to predict wind and scalar distribution in a treated orchard. The model is similar to that of Murlis and Jones (1981), but it applies to a uniform array of dispensers rather than a single point source and includes the effects of trees since their foliage has a major influence on the wind flow and hence the transport of pheromone. The model is one-dimensional, assumes variations only in the vertical direction, and neglects any effects of advection in the horizontal direction.

We consider the orchard canopy to be a spatially extensive, approximately steady distribution of sources and sinks, specifiable by a source distribution $S(z)$ (nanograms per cubic meter per second), which varies with the height z (meters) above the ground. We use the localized near-field theory of Raupach (1989) to calculate the resulting scalar concentration profile $C(z)$ (nanograms per cubic meter) produced by this source of density $S(z)$. The calculations assume a knowledge of the vertical profile for the mean horizontal wind speed $\overline{U}(z)$ (meters per second) and the associated turbulence properties $\sigma_W(z)$ (meters per second) and $t_L(z)$ (seconds) which represent, respectively, the corresponding velocity and time scales of the turbulence.

The localized near-field theory of Raupach calculates the scalar concentration $C(z)$ by decomposing the transport process into two parts, represented by a diffusive *far-field* component, $C_f(z)$, and a nondiffusive *near-field* component, C_n, such that

$$C(z) = C_f(z) + C_n(z) \tag{1}$$

The detailed structure of the C profile is almost entirely due to C_n, with C_f providing the background level and larger-scale variation.

Raupach's analytical theory is based on two approximations that are adequate in the inhomogeneous turbulence typically found in canopies (Raupach, 1989). First, the far-field, diffusive part, C_f, is found from the following integral formula (Raupach, 1989)

$$C_f(z) - C_f(z_R) = \int_z^{z_R} \frac{1}{\sigma_W^2(z')t_L(z')} \left[\int_0^{z'} S(z'')dz'' \right] dz' \tag{2}$$

where z_R is a reference height, which we take to be equal to two times the height of the canopy. The above integral is evaluated using an extended trapezoidal formula of order $(1/N^4)$ where N is the number of intervals (Press et al., 1988). The boundary value $C_f(z_R)$ is found from

$$C_f(z_R) = C(z_R) - C_n(z_R) \tag{3}$$

where $C(z_R)$ is the atmospheric concentration at the reference height, z_R, which must be specified a priori.

Secondly, the near-field nondiffusive part, $C_n(z)$ is determined from the following integral equation

$$C_n(z) = \int_0^{\infty} \frac{S(z')}{\sigma_W(z')} \left[k_n \left\{ \frac{z - z'}{\sigma_W(z')t_L(z')} \right\} + k_n \left\{ \frac{z + z'}{\sigma_W(z')t_L(z')} \right\} \right] dz' \tag{4}$$

where k_n is a kernel function, which, in practice, is evaluated from a simple analytic approximation, given by

$$k_n(\zeta) = k_n(-\zeta) = c_1 \ln(1 - e^{-\tau}) + c_2 e^{-\zeta}, \qquad \zeta > 0 \tag{5}$$

where $c_1 = -1/\sqrt{2\pi}$ and $c_2 = 0.5 - \pi^2/(2\sqrt{2\pi})$. The above equations are included here for the sake of completeness, but their derivation can be found in Raupach (1989). The near-field concentration declines rapidly with increasing distance from the source so that $C_n(z_R) = 0$, provided our reference height, z_R, is well above the tree tops. Here, we take z_R to be two times the tree height, h (meters), and we assume negligible atmospheric concentrations of pheromone above our reference height, so we set $C(z_R)$ equal to zero at the height $z_R = 2h$.

The following representation is made for the source distribution, $S(z)$. The magnitude of $S(z)$ is determined from the density of dispensers, ρ_d (number per cubic meter canopy ground area) and the average release-rate from each dispenser (nanograms per second). The profile shape of $S(z)$ is similar to the verti-

cal distribution of the dispensers. Here, we let $S(z)$ be normally distributed at a mean height z_d (meters) and with a standard deviation of σ_d (meters) because, in practice, some variation in dispenser height was found (see later).

The release rate from a single pheromone dispenser, dm/dt (nanograms per second) is approximated with the following equation

$$\frac{dm}{dt} = 0.52l(a + bT_a) \tag{6}$$

where l is the liquid length of the dispenser (millimeters) and T_a (degrees celcius) is the ambient air temperature. The empirical parameters, a and b, have been previously determined for our Shin Etsu dispensers (Bradley et al., 1995). The normal source–density distribution, the release of pheromone from a treated orchard, is approximated as

$$S(z) = \rho_d \frac{dm}{dt} \frac{1}{\sqrt{2\pi}\sigma_d} \exp\left[\frac{(z - z_d)^2}{2\sigma_d^2}\right]. \tag{7}$$

This distribution allows us, via the model, to change the density of the dispensers (ρ_d), their distribution within the canopy (z_d and σ_d), and their specific release-rate properties (l, a, b). It also allows us to modify the release-rate from the dispensers should there be any changes in the ambient temperature of the orchard air. An example of the profile $S(z)$ is shown in Figure 1.

For steady conditions in an extensive, uniform canopy, the source density profile, $S(z)$, and the vertical scalar flux density, $F(z)$, are simply related by

$$S(z) = dF/dz, \qquad F(z) = \int_0^{z'} S(z)dz' \tag{8}$$

The total vertical flux density from the orchard is given by $F(h)$ (nanograms per square meter per second), which is found simply by multiplying the release rate from a single dispenser, dm/dt, by the density of dispensers in the orchard, ρ_d. When advective edge effects are small, $F(h)$ is uniform above the tree canopy and represents the total loss of pheromone from the orchard. It follows from equation 8 that $F(h)$ declines with increasing depth into the canopy.

Having now specified the source density profile, $S(z)$, we next provide appropriate distributions for the mean wind speed and the associated turbulence within and above the orchard canopy. The mean wind speed within the canopy is approximated by an empirical exponential wind profile (Cionco, 1965), given by

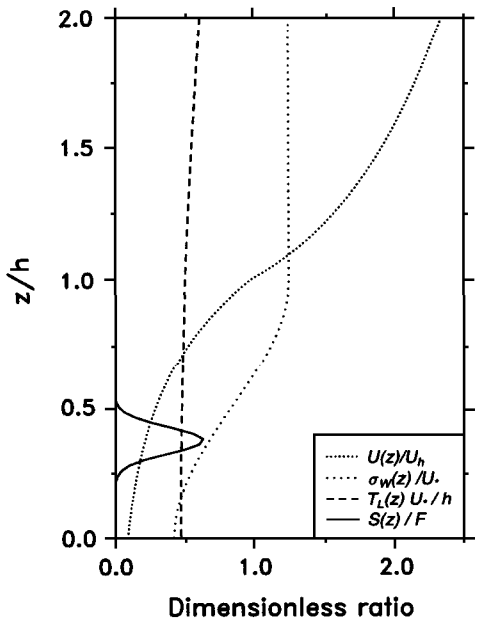

FIG. 1. Model inputs for the normalized profiles of the horizontal wind speed [$\underline{U}(z)/U_*$], the standard deviation of the vertical wind speed fluctuations [$\sigma_W(z)/U_*$], the Lagrangian time scale ($t_L U_*/h$), and source distribution [$S(z)$]. Here, the canopy height, h, the wind speed at tree-top height, U_h, and the friction velocity, U_*, are the normalizing length and velocity scales. Hence $Z/h = 0$ is the ground, 1 is the tree top, while the solid line represents the distribution of pheromone dispensers.

$$U(z) = U_h \exp[-\gamma(1 - z/h)], \qquad z < h \tag{9a}$$

where U_h is the wind speed at tree-top height, h (meters), and the coefficient γ tends broadly to increase with leaf area index, L_I (square meters of leaf area per square meter of ground area). Here γ was approximated as

$$\gamma = 0.89 + 0.8^* L_I \tag{9b}$$

based on the results of Raupach (1988), who collated data from a series of field and wind-tunnel studies. The leaf area index, L_I, was estimated from the tree dimensions and the foliage density, as described below.

Mean wind speed above the canopy was modeled as a standard log wind profile, given by

$$U(z) = \frac{U_*}{k} \ln[(z - d)/z_0], \qquad z \geq h \tag{10}$$

where U_* (meters per second) is the friction velocity, k is von Karman's constant (0.4), d (meters) is the zero-plane displacement height (=0.75 h), and z_0 (meters) is the roughness length (=0.13 h) (Monteith and Unsworth, 1990). The friction velocity is a simple turbulence scale used to relate the wind profile above a surface to the frictional drag per unit ground area on that surface. The usefulness of U_* will become obvious. We note that at the height of the canopy, where $z = h$, we assume that $U_h = 1.63\ U_*$. For modeling purposes, a mean wind speed profile was generated via equations 9 and 10, based on values of h and U_h, which were measured in the orchard at the same time as the air sampling was being carried out. Figure 1 gives an example of the vertical profile of wind speed $U(z)/U(h)$.

For the associated turbulence properties, we assumed profiles of σ_W and t_L consistent with the form suggested by Raupach (1989), although we modified some of the coefficients in order to account for a leaf area effect. The value of $\sigma_W(z)$ was assumed to be constant above the canopy, i.e., $\sigma_W(z) = a_1 U_*$ for $z \geq h$, where $a_1 = 1.25$ is the standard value in the surface layer well above the canopy (Raupach, 1988; Stull, 1988). Within the orchard canopy, the values of $\sigma_W(z)$ were decreased according to the expression

$$\frac{\sigma_W(z)}{U_*} = \tfrac{1}{2}(a_1 + a_0) + \tfrac{1}{2}(a_1 - a_0)\cos[\pi(1 - z/h)] \qquad z < h \tag{11}$$

with a_0 being the value of $\sigma_W(z)/U_*$ at the ground (Figure 1). We set a_0 equal to the maximum of $0.7 - 0.15^* L_I$ or 0.1, because canopy turbulence tends broadly to decrease with increasing canopy density. This value of a_0 was chosen to be consistent with the turbulence measurements of Green et al. (1995), made in a forest of widely spaced trees.

The profile for the Lagrangian time scale, $t_L(z)$ was taken to be the same as that proposed by Raupach (1989), namely

$$\frac{t_L(z)U_*}{h} = c_1\left(\frac{z - d}{h}\right) + \left(c_0 + \frac{c_1 d}{h}\right)\exp\left(\frac{-c_1 z/h}{c_0 + c_1 d/h}\right) \tag{12}$$

where $c_1 = k/a_1^2$ and $c_0 = 0.3$. The normalized profile, $t_L(z)U_*/h$, is nearly constant and assumes a value equal to about 0.4 at heights within and just above the tree tops (Figure 1). This implies that the Lagrangian time scale is about 3 sec for a wind speed of 1 m/sec at the top of 4.5-m-tall trees. Raupach (1989) showed the above turbulence profiles given by equations 8–12 are realistic in the upper and middle parts of a vegetative canopy.

The set of equations given above complete a simple analytical theory for

steady, advection-free conditions, assuming only vertical changes in the turbulent wind regime. We calculate the atmospheric concentration of pheromone from the superposition of $C = C_f + C_n$ with C_f given by equation 2 and C_n given by equation 4, both in terms of the source density distribution $S(z)$ and the turbulence properties $\sigma_w(z)$ and $t_L(z)$.

Experimental Sites. Experiments were carried out at Lincoln Springs apple orchard (Canterbury, New Zealand) in February and March 1996. This orchard was chosen because it approximates the ideal requirements of a flat, extensive, uniform canopy. Two treatments (1000 and 2000 dispensers/ha) were established in identical blocks of 4.5-m-tall apple trees, planted in rows, at a spacing of 2.2 m, with 4.4 m between each row. Each orchard block was approximately 1 ha in size and was enclosed by a shelter belt of poplar trees that was approximately 8 m in height. Near the center of each block ($\cong 15\ h$), the advective edge effects are likely to be small (Green et al., 1994), and so the vertical profiles of the wind turbulence and the corresponding profiles of atmospheric pheromone concentration should scale accordingly (see later).

For modeling purposes, the orchard blocks were described using the average tree height, h (meters), and the corresponding leaf area index, L_I (square meters of leaf area per square meter of ground area), of the orchard block, the latter being estimated from the average leaf area of an average tree (A_T = square meters of leaf per tree) and the planting density of the orchard (ρ_T = numbers of trees per square meter of ground area). We did not measure the leaf area of any trees, and so the following approximation was made. First, the crown volume, V_c (cubic meters) was estimated from a measurement of the crown dimensions of eight representative trees. For this calculation, we measured the tree height, the length of the crown, L_c (meters), and the radius of the crown, R_c (meters), at heights of 1.0, 1.5, 2.0, 2.5, and 3.0 m above the ground. We assumed a leaf area density value, ρ_L (square meters of leaf per cubic meters of crown volume], equal to 2.7 as being typical of pruned apple trees grown in the slender pyramid regime (J. Palmer, personal communication). Thus, the value of L_I was estimated from the product of ρ_L times V_c times ρ_T.

In one block, the dispensers (Shin-Etsu Fine Chemical Co., Tokyo) were evenly distributed at a density of 1000 point sources/ha, by placing the dispensers on a regular grid of 10-m × 10-m spacing. In the other block, a density of 2000 dispensers/ha treatment was achieved by placing a pair of dispensers at each grid point, these dispensers being placed just a few centimeters apart. Each dispenser was secured by tying it to the top wire supporting the trees, at a height of 1.88 ± 0.10 m in the 1000/ha block, and at a height of 1.74 ± 0.08 m in the 2000/ha block. The variation in dispenser height was determined by measuring the height above the ground of a sample of dispensers (N = 20) one week after application. This statistic was later used to provide parameters for the expression for the source distribution $S(z)$ via equation 3.

Pheromone Dispensers. The pheromone dispensers used here were obtained from the Shin-Etsu Chemical Co. and contained approximately 55 mg (*E*)-11-tetradecen-1-yl acetate (*E*11–14 : OAc), 2.5 mg *E*9,*E*11-tetradecadien-1-yl acetate (*E*9,*E*11–14 : OAc), 19.7 mg of the behavioral inhibitor (Rumbo et al., 1993) (*Z*)-11-tetradecen-1-yl acetate (*Z*11–14 : OAc), as well as stabilizers. The initial liquid length of the aged dispensers was measured in order to calculate the pheromone release rates via equation 6 (Bradley et al., 1995). Thereafter, the predicted pheromone loss was compared to the actual loss (as determined from the decrease in liquid length) measured on a weekly basis.

Meteorological Data. Air temperature, wind speed, and wind direction were recorded simultaneously at two different locations in the orchard. Air temperature was monitored by using screened temperature probes (model 107, Campbell Scientific), wind speed was monitored by using sensitive three-cup anemometers (A101M, Vector Instruments, Clywd, UK), and wind direction was monitored with a wind vane (W200P, Vector Instruments, Clywd, UK). At one location, fixed in the center of the 2000/ha plot, all measurements were made at tree-top height (h = 4.5 m) and the data recorded on a data logger (model CR10, Campbell Scientific, Logan, Utah) at a sampling rate of 0.5 Hz. At a second location, a mobile tower and data logging station were used to measure temperature and wind speed at three heights (corresponding to 0.5, 1, and 2 h). These measurements were synchronized with the air sampling of pheromone concentrations. Means and standard deviations of the meteorological data were calculated and recorded once every 15 minutes.

Air Sampling. Orchard air was sampled near the center of each plot by using a suction pump (Thomas, model 707CH820-194A, 60 liters/min) to pull air through filter traps. Three charcoal fiber filters (glass tubes, 8 × 0.8 cm ID, containing ~10 g activated charcoal fibers) (Shin-Etsu Chemical Co.) were used to trap the pheromone compounds. Each filter was connected via a hose and manifold to the pump. The flow rate through each filter was controlled individually at 12 liters/min with a flowmeter (Platon, B.R. Homersham Ltd., Christchurch, New Zealand). The sampling duration was 3 hr, so each sample collected the amount of pheromone contained in a volume of 2.2 m^3. Prior to each sampling, the filters were washed three times with 5 ml of *n*-hexane and then heated in a vented oven at 50°C for approximately 5 hr. In an initial study, the filters showed no measurable residues after they had been treated in this way.

The air samples were prepared for analysis in the following way. Charcoal filters were first extracted by percolation in the laboratory, on the same day, with three repetitions of 5 ml analytical *n*-hexane, with 20 ng of *n*-eicosane added as the internal standard. Solvent volume was reduced carefully, to approximately 0.5 ml, in a rotary evaporator (Bibby, RE 100, UK) set at 45°C and −0.7 bar. The volume was further reduced to 20–50 μl by placing the sample in a fume hood at room temperature. Finally, the sample was transferred to microvolume

inserts and sealed in autosampler vials. The variability associated with extraction and analysis was tested in an initial study and found to be low.

Gas Chromatography. Capillary gas chromatography (GC) was performed on an HP5890A gas chromatograph (Hewlett-Packard) equipped with a model 7673A autosampler and a splitless injector fitted with an Alltech Assoc. Carbowax Econo-Cap column (30 m × 0.32 mm ID, 25-μm-thick coating). The injector and flame ionization detector (FID) were held at 175°C and 225°C, respectively. The oven was held initially at 100°C for 1 min, then the temperature was increased at a rate of 5°C/min until it reached 195°C, and was maintained at this temperature for a further 5 min. Linear carrier gas (hydrogen) flow was ~30 cm/sec. Sample aliquots (2 μl from the pentane extracts) were injected, the injector being purged after 0.5 min. Instrument control, peak data collection, and integration were performed by an HP 3396 integrator. Initially, coupled GC and EAG detection and later, retention time were used for identification of the *E*11–14 : Ac peak. Concentration was estimated by using an internal standard (*n*-eicosane) and comparing the FID response of *E*11–14 : Ac to the response to the standard.

Model Predictions. A sensitivity analysis was carried out to examine how a change in the key model inputs influenced the mean atmospheric concentration of pheromone in a treated orchard. Predictions were made for a range of environmental conditions, including those that existed during the pheromone sampling occasions, and a range of operating conditions, including different wind speeds, dispenser deployment heights, air temperatures, dispenser ages, and dispenser densities. A standard reference condition was chosen: a tree height of 4.0 m, a tree spacing of 3.0 m, a crown length of 3.0 m, a crown radius of 1.5 m, a wind speed at tree-top height (U_H) equal to 2 m/sec, a dispenser height of 1.5 m (0.375 h) with a standard deviation of 0.2 m, an air temperature of 20°C, a dispenser liquid length of 100 mm, and a dispenser density of 1000/ha.

RESULTS

The model of Bradley et al. (1995) accurately predicted pheromone release from single dispensers over the three months of the experiment, by using just a measurement of the initial meniscus length and the accumulated air temperature. A good linear relationship was observed between actual and predicted changes in liquid length ($y = 1.0776x - 5.7417$, $r^2 = 0.99$). For periods during air sampling, the corresponding dispenser release rates were calculated from the Bradley et al. (1995) model by using the meteorological data recorded in the orchard at the same time.

For the purpose of modeling, the pheromone release from a single dispenser (nanograms per second) was expressed in terms of an equivalent flux density per

unit ground area, F (nanograms per square meter per second), by multiplying the single dispenser value by the density of dispensers in the orchard. Other inputs to the model included the vertical wind speed profile and the corresponding profiles of the turbulence properties, which are shown in Figure 1 in their scaled (normalized) form. Here, the canopy height, h (meters), the wind speed at tree-top height, U_h (meters per second), and the friction velocity, U_* (meters per second), are the normalizing length and velocity scales (Raupach, 1987). It is important to note that each of the wind properties are influenced by the distribution and density of foliage in the orchard, and we model these influences in equations 9–12. Any changes in the wind properties will have a significant impact on the predicted pheromone concentrations, as we will demonstrate.

First, however, we illustrate the mean concentration profile predicted for our reference conditions (see above) and we examine the relative contributions of the *near-field* and *far-field* components (Figure 2). The mean atmospheric concentration of pheromone within the canopy is predicted to be 0.5–2 ng/m^3,

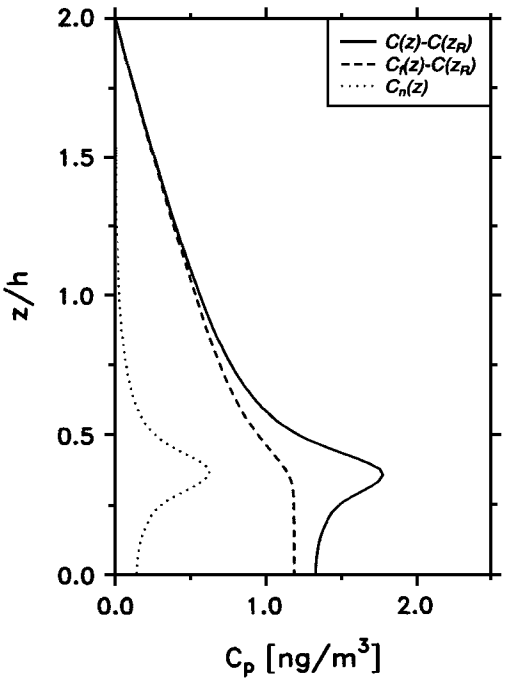

FIG. 2. Profiles of the near-field $C_n(z)$, far-field $C_f(z) - C(z_R)$, and total atmospheric concentration $C(z) - C(z_R)$ of pheromone in orchard air. Here $C(z_R)$ is the concentration at some reference height $z_R = 2h$, which is assumed equal to zero.

with a pronounced peak located at the height of the dispensers. This peak is due almost entirely to the *near-field* component, $C_n(z)$, while the *far-field* component, $C_f(z)$, provides for the background level and any larger-scale variation. The model shows clearly that dispenser application height has a major influence on pheromone concentrations. Furthermore, the predicted concentrations appear reasonable, since they are of the same order as other published results (Witzgall et al., 1996; Bäckman, 1996).

Indeed, when comparing the predicted concentrations against measurements from the air sampling, we found the model predictions were significantly correlated with the measured values ($P < 0.01$), although considerable scatter was evident in the measurements (Figure 3). For example, replicate measurements varied by a factor of two or more when made under the same meteorological conditions. The greatest scatter was evident at the level of the tree tops. Both the predicted and the measured values were within the range of 0.5–5.0 ng/m^3 across a wide range of environmental conditions. Possible reasons for the scat-

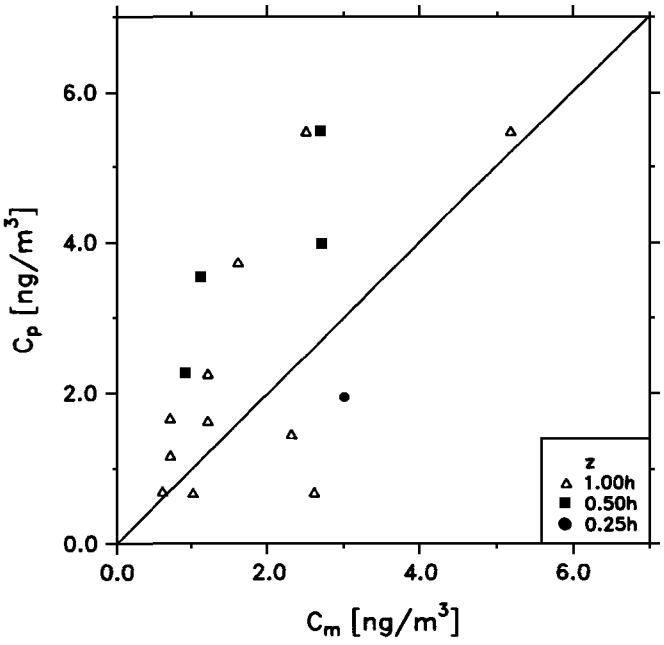

FIG. 3. Measured (C_M) and predicted (C_P) atmospheric pheromone concentrations at 0.25, 0.50, and 1.00 tree heights, following treatment of apple orchard blocks with polyethylene tubing dispensers (1000 or 2000/ha). ($C_p = 0.8 + 0.91\ C_m$, $r^2 = 0.36$, $P < 0.01$).

ter include problems of measuring low concentrations and the varying operating conditions that existed during the air sampling period. For example, the meteorological conditions and dispenser properties recorded at the time of sampling showed a wide range of conditions during the field measurements (wind speeds at the canopy top varied from 0.1 to 2.3 m/sec, air temperatures varied from 10 to 23°C, and dispenser contents ranged from 96.7–88.1 mm of pheromone liquid length). The impact of any advective edge effects due to the limited size of the orchard blocks would also have added to the scatter between measured and predicted pheromone concentrations. Notwithstanding this scatter, the results in Figure 3 show the model output is in the right range and they provide necessary experimental support for the simple model.

We continue by examining the influence of a range of environment conditions on mean concentration levels. First, the influence of wind speed is shown in Figure 4. As expected intuitively, this factor has a major impact on pheromone concentration. We predict a decline in concentration with increasing wind speed, which is almost linear, so that halving wind speed leads to a doubling in con-

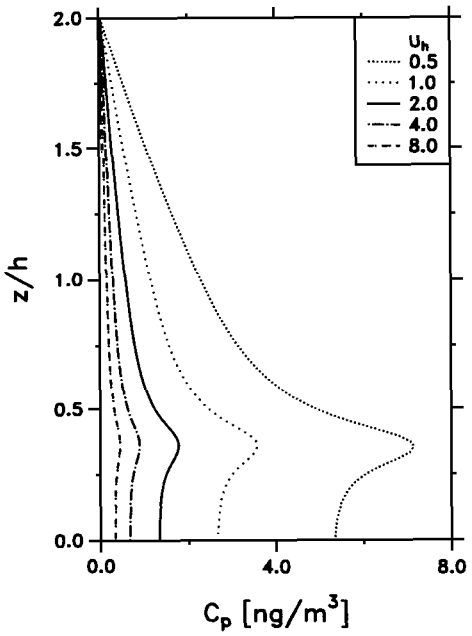

FIG. 4. The effect of different wind speeds, U, on the vertical profile of atmospheric pheromone concentrations (C_p) predicted in an orchard block treated with 1000 dispensers/ha. The standard model conditions are shown by the solid line in the Figures 5–7.

centration. According to our release-rate model, equation 6, the wind speed per se does not affect the source profile, and hence the total vertical flux density. Rather, it enhances the vertical turbulent transport so that more pheromone is transported out of the orchard, and hence lost to the system during more windy periods. Thus, diurnal variations in wind speeds would be expected to affect atmospheric concentration significantly. Because winds are generally lighter at night, we would expect higher concentrations during nocturnal periods, if wind were the only contributing factor. However, many other environmental factors also act to affect concentrations as we will demonstrate.

Meanwhile, environmental factors aside, we also expect that operational factors will influence the profile of mean concentration. The model results show that dispenser application height is important in determining not only where the concentration peak is located (Figure 2), but also in determining how much pheromone is retained within the orchard canopy (Figure 5). Peak concentrations decline with increasing dispenser height, mainly because of increased wind speed and increased turbulent mixing that occurs higher in the canopy (see Fig-

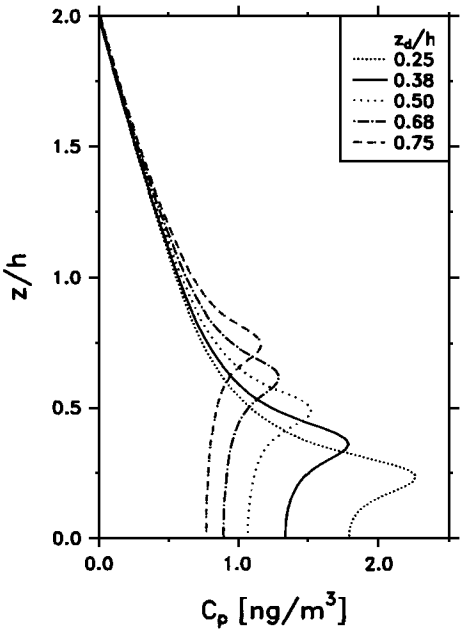

FIG. 5. The effect of different dispenser heights (z_s/h), on the vertical profile of atmospheric pheromone concentrations (C_p) predicted in an orchard block treated with 1000 dispensers/ha. The standard model conditions are shown by the solid line in the Figures 5–7.

ure 1). For the same environmental conditions, the concentration in the upper canopy can be increased beyond that which would be achieved by having lower dispensers, simply by placing the dispensers higher in the canopy. However, in practice, when the dispensers are placed higher, it may be necessary to increase their density in order to achieve the same level of protection, as inferred from the mean concentration levels. This result has important practical implications. The model results show that spreading the dispensers over a wider range of heights, for example, by varying the standard deviation of the dispenser height from 0.10 to 0.25 m, has only a secondary effect on mean concentration levels (Figure 6).

The influence of other environmental and operating factors can be summarized by presenting the concentration profiles in their normalized form (Figure 7). Here, the factor $C_* = F_*/U_*$ (nanograms per cubic meter) is a normalizing concentration, F_* (nanograms per square meter per second) being the total vertical flux density at the top of the canopy, i.e., $F(h)$, and U_* (meters per second)

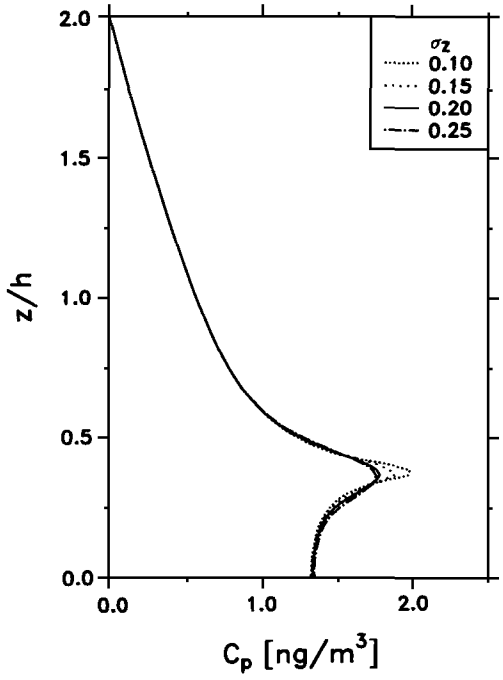

FIG. 6. The effect of a spread in the dispenser heights (σ_z) on the vertical profile of atmospheric pheromone concentrations (C_p) predicted in an orchard block treated with 1000 dispensers/ha. The standard model conditions are shown by the solid line in Figures 5–7.

being the corresponding friction velocity (Raupach, 1989). The scaling property of turbulent transport processes means that, for example, any changes in air temperature, dispenser density, or dispenser contents do not affect the shape of the concentration profile. Rather, they alter the pheromone flux from the dispensers. Thus, scaling by F_* removes the effect of the pheromone release rates, scaling by U_* removes the effect of the wind, and scaling by C_* produces a universal concentration profile for a given source height and profile shape (Figure 7).

Table 1 summarizes how F_* varies with air temperature and the density and age of the dispensers and can be used, in combination with Figure 7, to calculate the combined (multiplicative) effect of these variables on the profiles of mean concentration. For example, Table 1 shows that a doubling in air temperature from 10 to 20°C results in a twofold increase in F_* from 0.167 to 0.316 (nanograms per square meter per second). Thus, an increase in temperature from 10 to 20°C should result in a doubling of atmospheric concentrations. From Fig-

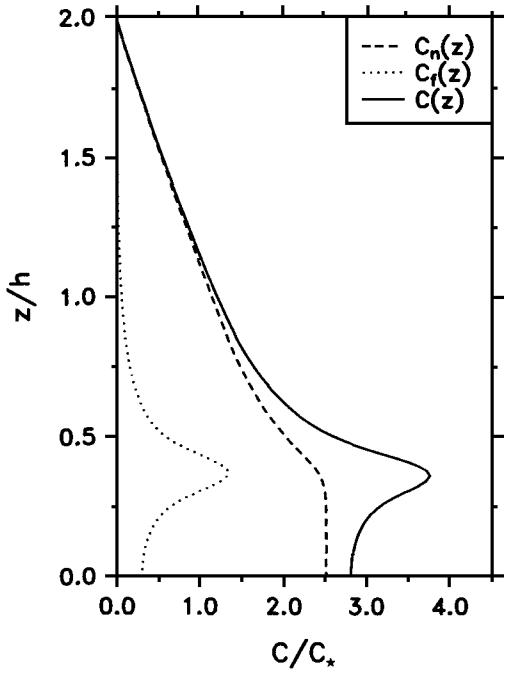

FIG. 7. Vertical profiles of the near-field, C_n, far-field C_f, and total C atmospheric concentration of pheromone, expressed in their normalized form. The factor $C_* = F_*/U_*$ is a normalizing concentration, F_* is the total vertical flux density, and U_* being the corresponding friction velocity (see text for details).

TABLE 1. INFLUENCE OF AIR TEMPERATURE, T_a, DISPENSER LENGTH, L_d, AND DISPENSER DENSITY ON VERTICAL FLUX DENSITY OF PHEROMONE, F_* RELEASED FROM TREATED ORCHARD[a]

T_a (°C)	L_d (mm)	ρ_d (per ha)	F_* (ng/m^2/s)
20	100	1000	0.316
10	100	1000	0.166
15	100	1000	0.241
25	100	1000	0.392
30	100	1000	0.467
20	50	1000	0.158
20	75	1000	0.237
20	125	1000	0.395
20	150	1000	0.474
20	100	250	0.079
20	100	500	0.158
20	100	2000	0.632
20	100	4000	1.265

[a]The standard reference conditions were used for the other environmental and operational variables (see text for details). The data in this table can be used with Figure 7 to calculate the actual concentrations by noting that $C_* = F_*/U_*$ and the friction velocity, $U_* = U_h/3 = 0.67$ m/sec.

ure 7 and Table 1, we calculate a peak concentration of about 0.92 ng/m^3 at 10°C, increasing to about 1.8 ng/m^3 at 20°C. Intuitively, we would expect a higher concentration in the orchard during periods of higher air temperature, simply because of the temperature response of the dispensers. The age of the dispensers also impacts linearly on the release rate of pheromone. Age is represented by the meniscus length, and this length alters F_* in the manner described by equation 6. Consequently, a doubling in the liquid length inside the tubing dispensers doubles the atmospheric concentration. We can conclude from Table 1 that raising the air temperature from 20 to 30°C has the same effect as increasing the dispenser meniscus from 100 to 150 mm. Finally, because there is also a linear relationship between dispenser density and the total flux density in the orchard, we also expect a linear, 1 : 1 increase in atmospheric concentration with increasing dispenser density.

DISCUSSION

The analytical model proposed here is a simple approach to demonstrate which factors are likely to influence atmospheric pheromone concentrations in treated orchards and thereby lead to a modification of insect behavior. The Lagrangian model was successful at predicting atmospheric pheromone concentra-

tions within the range of measured values. However, the comparison between observed and predicted values exhibited much scatter that we attribute mostly to the problems with our measurements. Measuring pheromone concentrations in air is technically difficult due to the minute amounts of pheromone involved (parts per billion). Furthermore, the physical properties of the pheromone molecules mean they are readily adsorbed onto surfaces, such as the glassware used during extraction and analysis, making difficult a complete recovery of any molecules trapped in the sample filters.

The model's ability to predict pheromone concentrations comparable to those measured in the field is, nevertheless, a useful progression towards overcoming the difficulty of obtaining direct measurements. Although more complex models may help to reduce the uncertainty, the model presented here is adequate to explain how environmental and operational factors influence mean pheromone concentrations in treated orchards. Other factors such as advection (e.g., upwind edge effects, possibly influenced by the poplar shelter trees) may also contribute to the scatter in our comparisons. This scatter may be enhanced at the tree tops because of the growth of an inner boundary layer above the canopy that enhances the turbulent transport process.

Other limitations of the model are acknowledged. In particular, the model does not account for all environmental factors, e.g., it ignores pheromone absorption and rerelease from leaf surfaces (Suckling et al., 1996), which may affect the source distribution in the orchard; it does not account for potential losses from the system (e.g., due to soil degradation); and it does not account for other sources in the orchard, such as any pheromones being released by the native moth population (likely to be minor). The model also assumes that a steady state exists over a given sampling period. However, rapid changes in pheromone concentration in orchards do occur, over seconds, as can be detected using electroantennogram recordings. These turbulent fluctuations are correlated with rapid changes in vertical wind speeds (Suckling and Karg, 1997), and they occur because clean air above is frequently swept down into the canopy, causing a sudden drop in pheromone concentrations. These vertical wind gusts originate in the turbulent boundary layer well above the tree tops and produce a similar flushing in both the air temperature and the humidity within the tree canopy (Denmead and Bradley, 1985).

Other researchers have measured the vertical profiles of pheromone concentrations with other dispenser formulations. Caro et al. (1980) sampled pheromone for 2–4 hr at three heights above the ground in a corn crop, and found that the pheromone concentrations were generally highest in the horizontal plane of the dispensers. Karg and Sauer (1995) also reported a peak in the plane of dispensers while measuring vertical profiles of mean pheromone concentration by using a field electroantennogram in a vineyard. Both these observations support our model results, which show a maximum concentration in the plane of the

dispensers, although we did not validate this profile here. Caro et al. (1980) also reported a sharp increase in pheromone concentrations with a decrease in wind speed, which also supports our model (Figure 5). Witzgall et al. (1996) measured pheromone concentrations of 1.3 ± 0.5 ng/m^3 by air sampling in plots of peas treated with Shin-Etsu polyethylene dispensers, and Bäckman (1996) reported similar values in apple orchards (although different dispensers were used with similar release rates). The pheromone concentrations they measured are in the same range as we modeled and measured in this study, which adds further support to our approach.

While several studies have indirectly investigated the influence of vertical concentration profile on mating disruption in orchards, complementary information on the atmospheric concentrations of pheromone was lacking. This information is important in characterizing the orchard environment and is key to improving our understanding of why mating disruption works. Suckling and Shaw (1992) reported that female lightbrown apple moths (*Epiphyas postvittana*) were more likely to mate at a height of 3.0 m in the canopy than at a height of 1.5 m where dispensers were located. Weissling and Knight (1995) reported that codling moth (*Cydia pomonella*) were present in higher numbers and mating was more successful in the upper canopy, so they recommended that pheromone dispensers should be deployed there.

The biological impact of different dispenser heights in treated orchards also has been examined by Lawson et al. (1996). They found no difference in the suppression of trap catch when dispensers were deployed at the top of the canopy compared to deploying half the dispensers each at the top and bottom parts of the canopy. We speculate that this null result may have been because of a similarity in the concentration profiles produced by the two treatments. A model such as the one presented here could be used to support this speculation and address why the two treatments gave similar trap catches.

While our simulations showed more pheromone is retained overall in the canopy by using lower dispensers, the corresponding peak concentrations that can be achieved by placing dispensers in the upper canopy may be important in practice. This observation, along with the manufacturer's recommendation to apply one third to one half of the dispensers in the upper canopy, suggests this to be true for insects that mate near the top of the tree (e.g., codling moth). For these insects, it may be more important to maintain a high concentration in critical areas of the upper canopy than to have more pheromone distributed evenly over all heights in the orchard.

So far, for modeling purposes, we have treated the dispensers as a horizontal plane source of pheromone. However, they are really point sources, and this distinction may have a significant effect on the efficacy of disruption, as well as the actual plume structure in treated orchards (Suckling and Angerilli, 1996). Interestingly, we note that a twofold (Suckling and Shaw, 1992) and a

fourfold (Suckling et al., 1994; Lawson et al., 1996) variation in the density of dispensers appears to have had little measurable impact on the efficacy of disruption, as measured both by traps and by tethered females. This apparent lack of a response may be due to confounding by other factors, such as wind speed and the uncertainty associated with these biological methods. In this case, a model of the system could be helpful in resolving such discrepancies.

While the model presented here determines the mean vertical profile of pheromone concentrations, there are still many unanswered questions that we hope to address in further models we are currently developing. In particular, there is a need for a better understanding of advective effects on the horizontal distribution of pheromone near the edges of treated areas. There is also a need to incorporate the influence of foliage absorption and rerelease of pheromone, since behaviorally important amounts of pheromone are both absorbed and released by apple foliage in orchards treated with dispensers (Karg et al., 1994; Suckling et al., 1996). To determine whether and how a particular moth will respond, both the concentration of pheromone and the time of exposure should be known, since a brief exposure to a high concentration may or may not elicit the same response as a longer exposure to a low concentration. In the future, we should also address fluctuations in concentration since the peak fluctuations may be many times greater than the mean values (Aylor, 1976).

In closing, the parameters required to run the model (canopy profile description, meteorological measurements, and a dispenser release rate model) are simple to generate, and so the outlook is promising for reliably calculating pheromone concentrations under field conditions in other treated crops.

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ALLELOCHEMICAL POTENTIAL OF *Metopium brownei*

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Abstract—*Metopium brownei* is a tree that grows in coastal tropical forests along the Gulf of Mexico and in the Yucatan Peninsula. This medicinal species produces a strongly irritant exudate, and sometimes forms pure populations favored by fire. The bioactivity of the aqueous leachates, organic extracts (leaves, bark, and wood), and mixtures of urushiols and flavonoids from *M. brownei* were evaluated on the growth of two plants: *Amaranthus hypochondriacus* and *Echinochloa crusgalli*, and four phytopathogenic fungi: *Fusarium oxysporum*, *Helminthosporium* sp., *Alternaria* sp., and *Pythium* sp. Alkylcatechols (urushiols) were isolated from an acetone extract of the bark. Dihydroquercetin and eriodictyol were isolated from the chloroform–methanol extract of the wood. In addition, masticadienoic acid was isolated from the leaves. The aqueous leachates, organic extracts, and the mixtures of flavonoids and urushiols were inhibitory to the growth of test plants and phytopathogenic fungi. The allelochemical role of the bioactive compounds from *M. brownei* is discussed in relation with other results reported in some studies on Anacardiaceae family and *M. brownei*.

Key Words—*Metopium brownei*, Anacardiaceae, dihydroquercetin, eriodyctiol, masticadienoic acid, urushiols, alkylcatechols, allelochemicals, allelopathics.

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INTRODUCTION

Metopium brownei (Jacq.) Urban. (Anacardiaceae), commonly known as "chechém negro," "boxchechém," "kabal chechém," and "madera negra venenosa," is a tree (8–25 m high) that grows in tropical forests on the Gulf of Mexico coast from Veracruz to the Yucatan Peninsula. The genus *Metopium* is one of the most important in some plant communities (Smith and Vankat, 1992). *M. brownei* is considered a representative arboreal species of the Anacardiaceae that can grow in flooding areas. *M. brownei* is very resistant to fire, and sometimes forms pure populations favored by fire. It has a thin, gray–brown bark, composite smooth leaves (3–7 oval folioles, 3–8 cm), green yellowish small inconspicuous flowers in axillary panicules, and characteristic brilliant red drupe fruits (1–2 cm) (Cabrera-Cano et al., 1982).

Most of the leaves of *M. brownei* fall between April and May, simultaneous with flowering (Rzedowsky, 1978; Cabrera-Cano, et al., 1982; Ramamoorthy et al., 1993). These leaves are almost free of pathogens and herbivorous damage. Soils covered with fallen leaves of this tree show very scarce growth of understory plants. *M. brownei* is used medicinally as an antiviral, cathartic, diaphoretic, antiinflammatory, and sedative; this tree also has economic importance in the region. Its hard, beautiful wood is utilized in the fabrication of wood floors and staves (Martinez, 1979; Amo, 1979; Mendieta and Amo, 1981; Gómez-Pompa et al., 1991).

In a previous study (Rivero-Cruz et al., 1997), the composition of *Metopium brownei* urushiol was established. Three 3-*n*-pentadec(en)ylchatechols (urushiols) with antifungal properties and toxicity to brine shrimp (LC_{50} = 89.13 μg/ml) were identified. Rivero-Cruz et al. (1997) suggested that urushiols could be of importance in the defense mechanisms of the plant by preventing the attack of some fungi and bacteria.

The present study was undertaken to continue the study on the secondary compounds produced by *M. brownei* and to examine the effect of some of them on the growth of plants and fungi, and their possible ecological role.

METHODS AND MATERIALS

Plant Material. The aerial bark and wood of *M. brownei* were collected in the Municipio of Tulum, Quintana Roo, in November 1995 (voucher specimen AL95-16. National Herbarium, MEXU, Instituto de Biología, UNAM). The plant was dried at 30°C. Fallen leaves were collected at the El Eden Ecological Reserve, Quintana Roo, Mexico in June of 1996.

Preparation of Aqueous Leachates. Aqueous leachates of leaves, fallen leaves, bark, and wood were prepared by soaking the dry plant material in dis-

tilled water (2% w/v) for 4 hr. Leachates were filtered and their osmotic pressure measured in a freezing-point osmometer (Osmette A. Precision Systems, Inc.) in order to prevent the negative osmotic effects of a highly concentrated solution on germination and growth of the test plants (Anaya and Rovalo, 1976).

Bioassays with Seeds. Aqueous leachates were mixed (1 : 1) with 1.5% pure agar to obtain a 1% w/v test solution. Pure agar (0.75%) was used as the control. Germination and seedling radicle growth bioassays for the aqueous leachates were carried out according to previously published procedures (Anaya et al., 1990) with *Amaranthus hypochondriacus* L. (Amaranthaceae) and *Echinochloa crusgalli* (L.) Beauv. (Poaceae). The seeds of *A. hypochondriacus* were purchased from Mercado de Tulyehualco, Mexico, D.F., and those of *E. crusgalli* from Valley Seed Service, Fresno, California. The bioassays were set up in 5-cm Petri dishes, 10 seeds per dish, following a complete random block design with four repetitions per treatment. Petri dishes were kept in darkness at 27°C (4). Germination and radicle growth were measured after 24 hr for *A. hypochondriacus* and 48 hr for *E. crusgalli.*

Preparation of Organic Extracts. Organic extracts were prepared by macerating the dry leaves, bark, and wood of the plant in hexane, chloroform, methanol, or chloroform–methanol (1 : 1) at room temperature, for 72 hr. After evaporation of the solvent, each extract was resuspended in order to obtain 50, 100, and 200 μg/ml solutions.

Bioassays with Seeds. Bioassays with organic extracts were set up in the same conditions as with the aqueous leachates, but filter paper was used instead of agar. Filter paper in Petri dishes was impregnated with 1.5 ml of the solutions at 50, 100, and 200 μg/ml, and after total evaporation of the solvent, the paper was moistened with 1.5 ml of distilled water. Test seeds for these bioassays were *A. hypochondriacus* and *E. crusgalli.* Following this same procedure, the crude extract of urushiols from the bark and the crude extract of flavonoids from the wood of *M. brownei* were tested at 50, 100, 200, 700, and 1000 μg/ml on the same species of seeds. In this last bioassay, a positive control of 2,4-D was used.

Bioassays with Phytopathogenic Fungi. Organic extracts and a mixture of urushiols and flavonoids were tested on the radial growth of *Pythium* sp., *Alternaria* sp., *Fusarium oxysporum,* and *Helminthosporium longirostrastum.* The bioassays were carried out in 9-cm Petri dishes by procedures previously described (Castañeda et al., 1992; Hamburger and Cordell, 1987). Organic extracts and flavonoids were tested at 50 and 200 μg/ml. Urushiols were tested at 50, 100, 150, 250, 500, and 700 μg/ml, and Captan, a commercial fungicide, was used as a positive control. The measurements were made after 72 hr of incubation. Bioassays were set in a complete random block design with three repetitions per treatment.

All data were analyzed with ANOVA and Tukey's test.

Phytochemical Experimental Procedures. Brine shrimp lethality test (BST)

bioassays were used as a tool for the biodirected fractionation of *M. brownei* as described by Anderson et al. (1991). Melting points were determined by means of a Fischer Johns apparatus and were uncorrected. IR spectra were measured on a Perkin Elmer 599B spectrometer with KBr disks. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR-3005 instrument operating at 300 MHz and 75 MHz, respectively, in $CDCl_3$, C_5D_5N, or C_6D_6 with TMS as an internal standard. UV spectra were taken on a Shimadzu UV 160U spectrophotometer.

Isolation of Masticadienonic Acid from Leaves of M. brownei. Dried and ground leaves (5.045 kg) were extracted at room temperature with $CHCl_3$–MeOH (1 : 1). After filtration the extracts were combined and concentrated in a vacuum. The resulting residue (396.2 g, BST LC_{50} > 1000 μg/ml) was submitted to open column chromatography on silica gel with step gradient elution: hexane, hexane–$CHCl_3$ (9 : 1, 8 : 2, 7 : 3, 6 : 4, 1 : 1, 3 : 7), $CHCl_3$, and $CHCl_3$–MeOH (99 : 01, 95 : 5, 9 : 1, 9 : 2, 7 : 3, 6 : 4, 1 : 1). A total of 274 fractions (1 liter each) were collected and pooled based on TLC profiles to yield 13 major fractions (FL_1–FL_{13}). FL_3 was subjected to repeated silica gel column chromatography to yield 300 mg of masticadienoic acid, identical to a standard sample previously isolated from *Ampypterygium adstringens* (Navarrete, 1986).

Isolation of Dihydroquercetin and Eriodyctiol from Wood of M. brownei. The air-dried pulverized plant material (1.3 kg) was extracted with a mixture of $CHCl_3$–MeOH (1 : 1). The resulting extract was concentrated on a rotavaporator to give a residue (30 g, BST LC_{50} = 214 μg/ml). This residue was subjected to open column chromatography on silica gel (300 g) and eluted with a gradient of hexane and $CHCl_3$–MeOH. A total of 189 fractions was combined on the basis of their TLC patterns to yield 10 primary fractions (FW_1–FW_{10}). Biological testing of the primary fractions showed that FW_4 was toxic (BST LC_{50} = 181.98 μg/ml). Similarly, using the direct bioautographic TLC assay for detecting antifungal substances, only fraction FW_4 contained the fungitoxic activity. FW_4 (1.92 g) was rechromatographed on a flash silica gel (45 g) column by using benzene with increasing amounts of ethyl acetate to yield dihydroquercetin (80 mg), mp 235–240°C [lit. mp, 240–242°C] and eriodyctiol (8 mg), mp = 265–267°C [lit. mp, 267°C].

RESULTS

In Figures 1 to 12, only the most significant results of the present study are shown.

Bioassays with Seeds. The aqueous leachates (1%) of fresh and fallen leaves and bark inhibited radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli*, but the aqueous leachate of wood did not produce any effect on radicle growth of either target species (Figure 1). All aqueous leachates

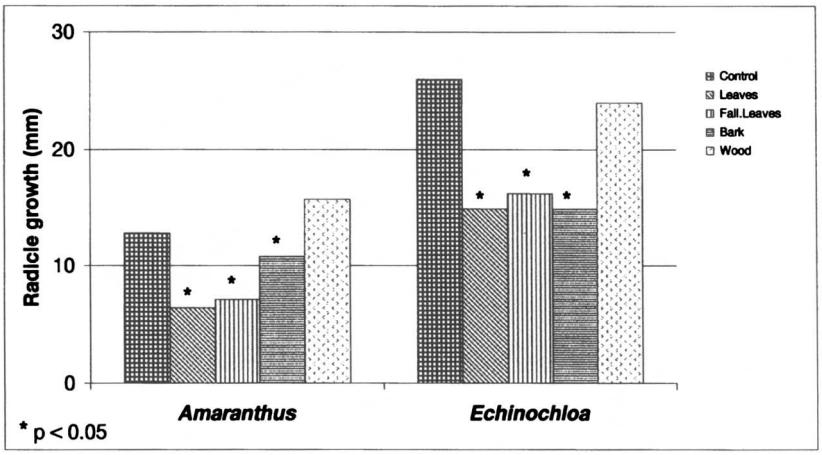

FIG. 1. Effects of aqueous leachates leaves, fallen leaves, bark, and wood of *Metopium brownei* on the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli* ($N = 4$).

(1%) showed very low osmotic pressure values (14–35 mosm/liter). These osmotic pressure values do not affect the growth of the seeds as previous studies have shown (Anaya and Rovalo, 1976). The results of bioassays carried out with hexane extracts of leaves are shown in Figure 2. The hexane extract of leaves inhibited radicle growth of *A. hypochondriacus* at 100 and 200 μg/ml, but had a nonsignificant effect on *E. crusgalli*. Compared with the effect of the aqueous leachate of leaves, the hexane extract of leaves had a less inhibitory effect than the aqueous leachate of leaves (Figure 1). Methanol and chloroform extracts of leaves had no effect on either test plant species (results not shown). Frequently, the phytotoxic effect of plant aqueous leachates is lost when the bioactive compounds from the plant are extracted with organic solvents. Figure 3 shows the effects of the chloroform–methanol (1 : 1) extracts from the bark and the wood at three concentrations on the radicle growth of both test plants. The extract had a significant inhibitory effect on the radicle growth of *E. crusgalli* at 100 and 200 μg/ml but no effect on *A. hypochondriacus*. The chloroform–methanol extract from the wood inhibited radicle growth of *E. crusgalli* only at 200 μg/ml.

Phytochemical fractionation of a $CHCl_3$–MeOH extract of the leaves of *M. brownei* allowed the isolation and identification of masticadienoic acid (Figure 4c). This compound has no effect on the radicle growth of *Amaranthus* and *Echinochloa* (results not shown).

The $CHCl_3$–MeOH extract from the wood was fractionated by column chromatography to render a flavonoid mixture, and eriodyctiol and dihydroquercetin were isolated (Figure 4a and b). These compounds were identified

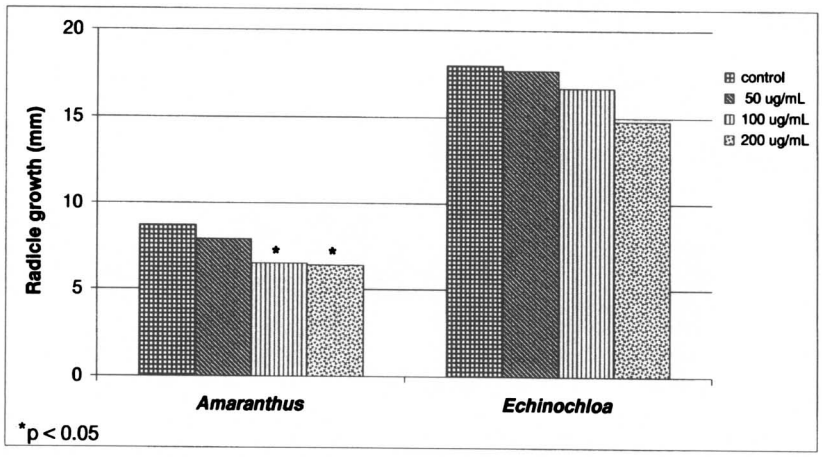

FIG. 2. Effects of the hexane extract of leaves of *Metopium brownei* (50, 100, and 200 μg/ml) on the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli* ($N = 4$).

by spectral means (Wagner et al., 1976; Shen and Chang, 1993). The acetone extract from the stem bark was subjected to column chromatography on silica gel to yield an urushiol fraction that contained 3-pentadecylcatechol (Figure 5a), 3-(10′*Z*-pentadecenyl)catechol (Figure 5b), and 3-(10′*Z*, 13′*E*-pentadecadi-

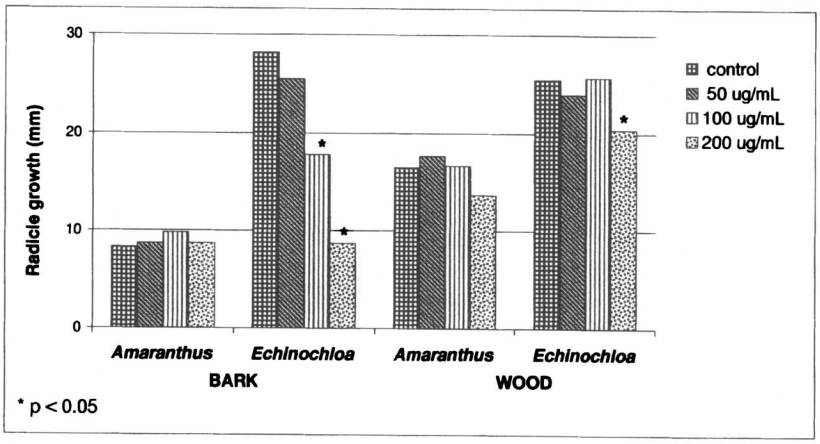

FIG. 3. Effects of organic extracts of bark and wood of *Metopium brownei* (50, 100, and 200 μg/ml) on the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli* ($N = 4$).

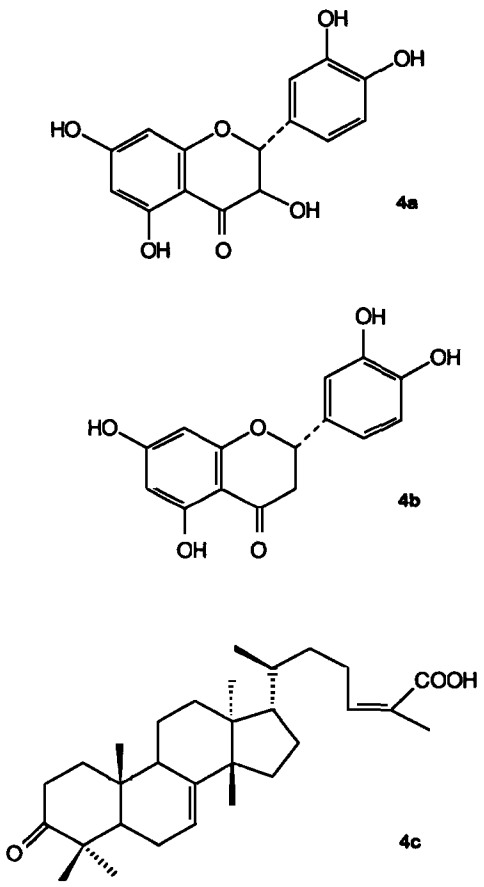

FIG. 4. Structures of dihydroquercetin (a) eriodictyol (b), and masticadienoic acid (c) from *Metopium brownei*.

enyl)catechol (Figure 5c). The separation and identification of these alkyl catechols were previously described (Rivero-Cruz et al., 1997).

Figure 6 shows the effects of five concentrations of flavonoids and urushiols, and 2,4-D on the radicle growth of *A. hypochondriacus*. Flavonoids inhibited radicle growth at 200–1000 μg/ml. Linear regression analysis indicated an $IC_{50} > 1000$ μg/ml. Urushiols inhibited *Amaranthus* radicle growth only at 700 and 1000 μg/ml ($IC_{50} > 1000$ μg/ml) and had a less inhibitory effect compared with flavonoids. 2,4-D used as a positive control had a strong inhibitory effect on *Amaranthus* at 50 μg/ml and totally inhibited radicle growth at 100 μg/ml ($IC_{50} = 16.1$ μg/ml).

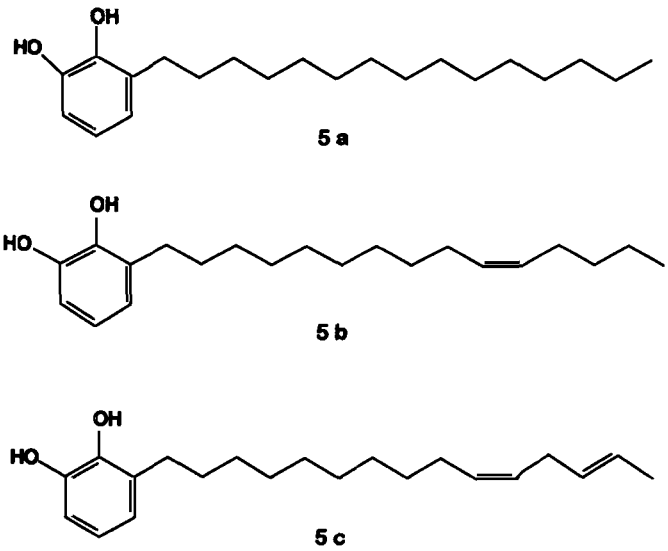

FIG. 5. Structures of urushiols, 3-pentadecylcatechol (a), 3-(10′*Z*-pentadecenyl)catechol (b), and 3-(10′*Z*, 13′*E*-pentadecadienyl)catechol (c) from *Metopium brownei* bark.

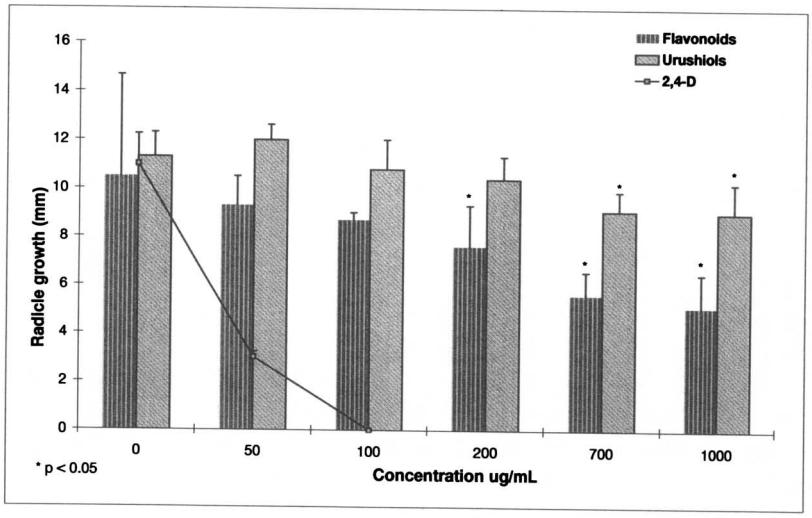

FIG. 6. Effects of flavonoids and urushiols from *Metopium brownei* at six concentrations, and 2,4-D on the radicle growth of *Amaranthus hypochondriacus* ($N = 4$).

Figure 7 shows the results of the bioassays testing flavonoids, urushiols, and 2,4-D on the radicle growth of *E. crusgalli*. At low concentrations (50–200 μg/ml) flavonoids did not inhibit radicle growth. Flavonoids inhibited only at 700 and 1000 μg/ml (IC_{50} = 628.3 μg/ml). Urushiols inhibited *Echinochloa* from 50 to 1000 μg/ml (IC_{50} > 1000 μg/ml). However, the effect of flavonoids at high concentrations was stronger than the effect of urushiols. *Echinochloa* was more resistant to 2,4-D (IC_{50} = 35.2 μg/ml) than *Amaranthus*.

Bioassays with Phytopathogenic Fungi. A methanol–chloroform (1 : 1) extract from the leaves of *M. brownei* had a significant but moderate inhibitory effect only on *Fusarium* radial growth (results not shown). The methanol extract of the bark inhibited the radial growth of *Helminthosporium* and *Pythium* (50 and 200 μg/ml), and also inhibited *Fusarium growth* at 200 μg/ml (Figure 8). The methanol extract of the wood of *M. brownei* inhibited *Pythium* (200 μg/ml) and *Helminthosporium* (50 and 200 μg/ml) (Figure 9). A significant inhibitory effect of two concentrations of flavonoids was observed on *Pythium* and *Alternaria* at 200 μg/ml (Figure 10).

All concentrations of urushiols produced a significant inhibitory effect on the radial growth of *Fusarium* (30–64% inhibition) (IC_{50} = 150 μg/ml) (Figure 11). The fungicide Captan, used as a positive control in this bioassay, had higher inhibitory effect compared with the urushiols at low concentrations (50 and 100

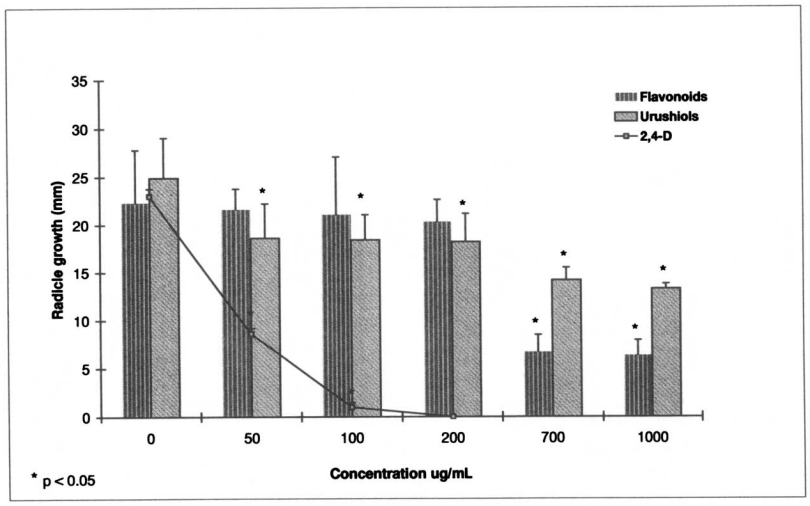

FIG. 7. Effects of flavonoids and urushiols from *Metopium brownei* at six concentrations, and 2,4-D on the radicle growth of *Echinochloa crusgalli* (N = 4).

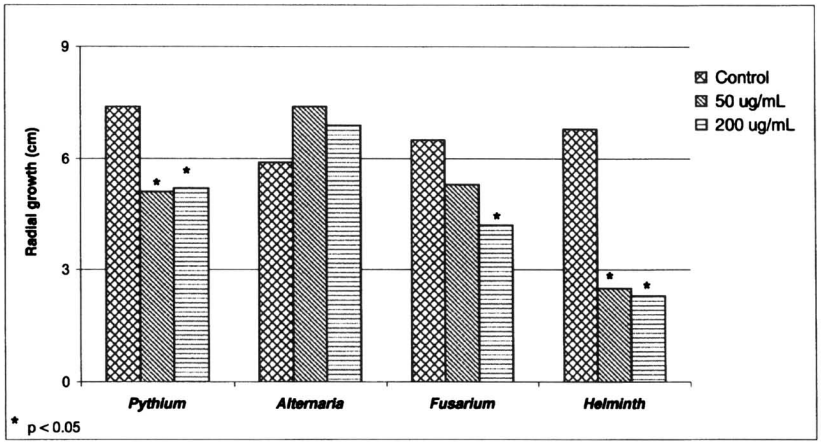

FIG. 8. Effects of methanol extract (1 : 1) of bark from *M. brownei* at 50 and 200 μg/ml on the radial growth of four phytopathogenic fungi (N = 3).

μg/ml) and high concentrations (500 and 700 μg/ml), but both had a very similar effect at 150 and 200 μg/ml (IC_{50} = 85.86 μg/ml).

Helminthosporium was also significantly inhibited by the urushiols at all concentrations tested (IC_{50} = 224.9 μg/ml) (Figure 12). The effects of Captan and urushiols were very similar on the growth of *Helminthosporium* from 150 to

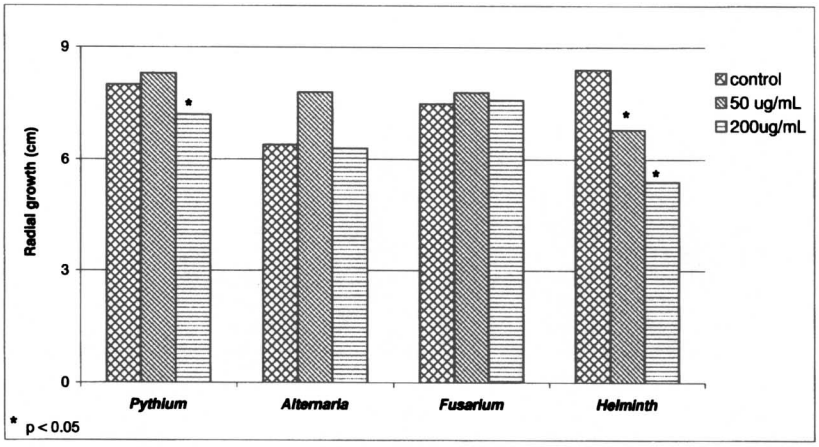

FIG. 9. Effects of the methanol extract (1 : 1) of wood from *M. brownei* at 50 and 200 μg/ml on the radial growth of four phytopathogenic fungi (N = 3).

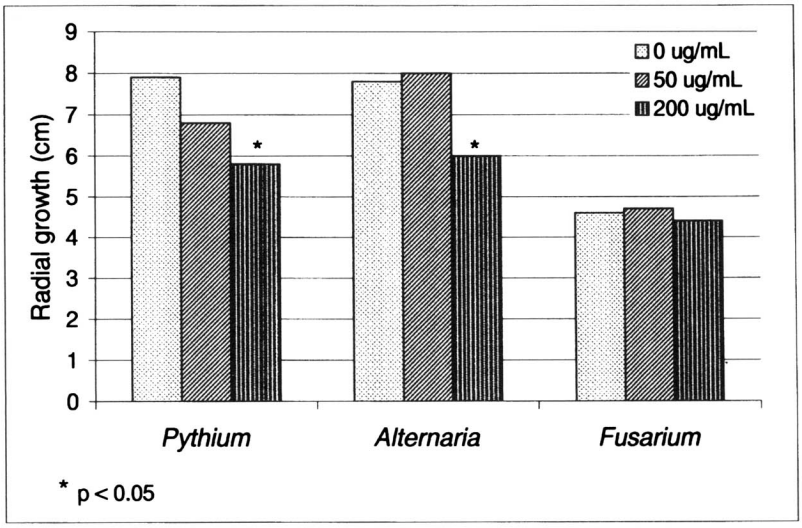

FIG. 10. Effects of flavonoids from *M. brownei* wood at 50 and 200 μg/ml on the radial growth of three phytopathogenic fungi (N = 3).

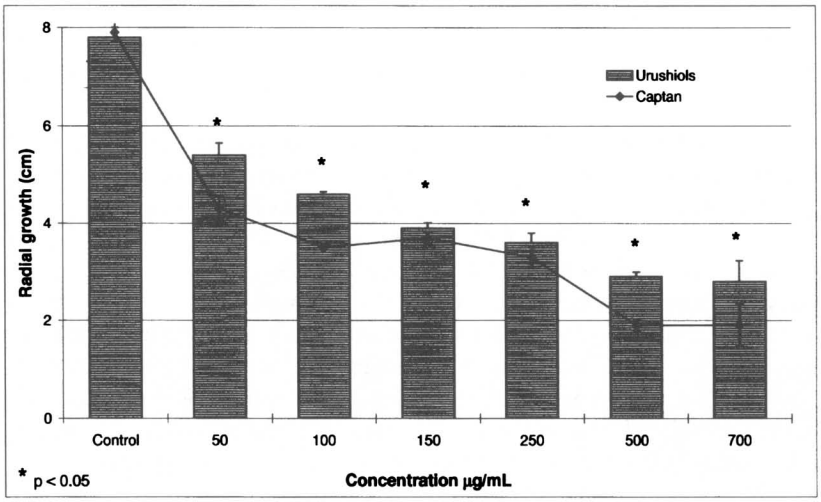

FIG. 11. Effects of urushiols from *M. brownei* bark at six concentrations on the radial growth of *Fusarium oxysporum* (N = 3).

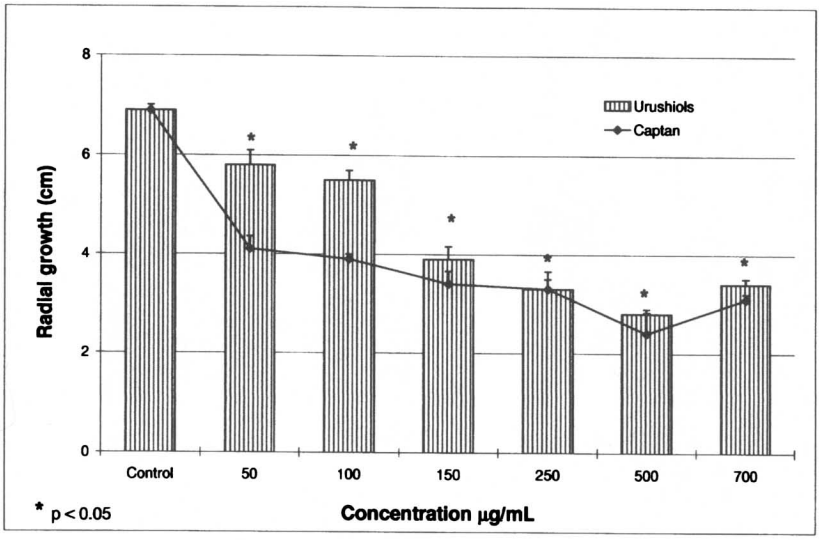

FIG. 12. Effects of urushiols from *M. brownei* bark at six concentrations on the radial growth of *Helminthosporium longirostratum* ($N = 3$).

700 µg/ml (IC_{50} = 155.3 µg/ml). Compared with *Fusarium*, *Helminthosporium* was more resistant to urushiols, particularly at lower concentrations.

DISCUSSION

The great majority of flavonoids, the best-known group of polyphenols, comprises some 4000 structures of plant origin. Most phenolics are toxic to living organisms. Eriodictyol and dihydroquercetin in *M. brownei* belong to the group of minor flavonoids (Harborne and Baxter, 1993) that have more limited natural distribution than anthocyanins, flavones, and flavonols. Eriodictyol possesses antibacterial activity. It shows growth inhibitory activity against larvae of *Heliothis zea*, and induces nodulation gene expression in *Rhizobium leguminosarum* in the symbiosis between this bacterium and its legume host, *Pisum sativum*. Dihydroquercetin (Taxifolin) has many biological activities. It is antimicrobial, antiinflammatory, antihepatotoxic, and antioxidant. It stimulates RNA synthesis, inhibits different enzyme activities, and inhibits the growth of *Heliothis zea* (Harborne, 1991). The influence of plant flavonoids on herbivore feeding (Coleoptera: Chrysomelidae), mainly *Rhus* spp., has been noted (Furth and Young, 1988). In the present study we observed a phytotoxic activity of flavonoids from the wood of *M. brownei* on radicle growth of *A. hypochondri-*

acus and particularly on that of *E. crusgalli*. Flavonoids also had a significant inhibitory effect on the radial growth of *Pythium* sp. and *Alternaria* sp.

The literature on Anacardiaceae shows that regardless of the geographical locality, different species of this family cause problems for mammals, including humans. The most common effect is allergic dermatitis. Poison ivy, mangoes, cashews, an indigenous Anacardiaceae at the Southern Transvaal, *Smodingium argutum*, and many other species, including *M. brownei*, are involved in health problems (Geller, 1989; Gorst-Allman et al., 1987; Wahlberg and Lovell, 1996; Rivero-Cruz et al., 1997). Sometimes pistachio nuts can provoke anaphylaxis, but the allergens involved are still poorly characterized (Fernandez et al., 1995). Rivero-Cruz et al., (1997) confirmed that *Metopium brownei* produces alkenyl phenols (with a mono-, di-, or triunsaturated side chain), as do most of the allergenic Anacardiaceae species that have been studied. In the Navassa Islands, West Indies, *M. toxiferum* causes contact dermatitis in goats (Brown et al., 1973). It has been demonstrated that urushiols inhibit arachidonic acid metabolism and could be used as anti-allergic agents in hyposensitization therapy (Gross et al., 1975; Watson et al., 1981, 1983; Duke, 1985; Harborne and Baxter, 1993).

Saps of Anacardiaceae contain mixtures of catechols and are used to produce lacquer ware. Japanese lacquer originates from the sap of *Toxicodendron vernicifluum* (syn. *Rhus verniciflua*), the lacquer tree (Vogl et al., 1995; Vogl and Mitchell, 1996; Qin et al., 1996). The antimicrobial effects of the saps probably are related with their content of catechols. The gum exudate of the cashew tree (*Anacardium occidentale*) inhibited growth of some fungi and bacteria, and the gum also prevented oviposition and reduced the number of larvae of the Bruchid beetle *Callosobruchus maculatus*. The feeding of the larvae of the chrysomelid beetle *Crimissa cruralis* was also strongly affected by the gum (Marques et al., 1992). Reyes-Chilpa et al. (1987) found that extractives from different tree species, including Anacardiaceae *Astronium graveolens*, are primarily involved in heartwood resistance to brown rot fungus *Lenzites trabea*. Several studies also showed that the presence of antifungal compounds in *Rhus glabra* branches and mango fruits, and the effect of phytopathogenic fungi on mangiferin production in the twigs of mango (Droby et al., 1985, 1986; Chakrabarty and Ghosal, 1985; McCutcheon et al., 1994). Furthermore, under field conditions, water extracts of *Metopium brownei* diminished the damage produced to a corn crop by the fall armyworm, *Spodoptera frugiperda* (Gonzalez-Gaona and Lagunes-Tejeda, 1986). These results, including the fungitoxic activity of *M. brownei* urushiols that we observed in the present study, suggest an active role for bioactive metabolites of the gum, latex, and sap of various Anacardiaceae in defense mechanisms of the plants.

Anacardiaceae is one of the plant families from which the native bees *Melipona rufiventris paraensis* and *Frieseomelitta varia* collected pollen in Amazonia (Marques-Souza et al., 1995). A study of nectar sources of European and

Africanized honey bees (*Apis mellifera* L.) in the Yucatan Peninsula during the wet and dry seasons showed that the most common species in honey samples was *M. brownei* (Villanueva, 1994). In addition, *M. toxiferum* (poison-wood) is one of the five dominant species of the diet of nestling white-crowned pigeons (*Columba leucocephala*) from July through September (Bancroft and Bowman, 1994). These examples call attention to different ecological adaptations of several organisms in their interactions with Anacardiaceae species. Different bioactive secondary compounds (including flavonoids and urushiols) are involved in these biotic interactions. The bioactivities differ depending on the type of biotic relation that the plant establishes.

Coley and Barone (1996) suggested that leaves of tropical forests have both higher overall levels of defense and a greater diversity of defense compared to their temperate counterparts. This greater commitment to defense is an evolutionary response to elevated pressure from herbivores. In the tropics, mature leaves are long-lived and must therefore be resistant to both abiotic and biotic damage. These defense mechanisms are present in other organs of the plant, such as stem bark, that has great herbivory pressure, mainly from microorganisms. This is also true for *M. brownei*, whose bark possesses defensive urushiols that can be effective on different herbivores (microorganisms to mammals) and they have phytotoxic effects.

Urushiols and flavonoids, among other secondary metabolites of *M. brownei*, afford different kinds of protection to the plant, as antibiotics, antifeedants, allergens, and probably allelopathics. Further studies on biotic interactions between *M. brownei* and their neighboring plants, endophytic and pathogen fungi, insects, and also vertebrates, will shed more light on the ecological role of the different bioactive secondary metabolites that this important Anacardiaceae species of the Peninsula of Yucatan, Mexico produces.

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FRAGRANCE COLLECTION, STORAGE, AND ACCUMULATION BY INDIVIDUAL MALE ORCHID BEES

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Abstract—Individually marked males of two species of *Euglossa* were sighted repeatedly and over considerable periods of time (up to 44 days) at artificial fragrance baits exposed on Barro Colorado Island (BCI), Panama. Individuals switched between different bait chemicals that are attractive for the respective species, and no bait preferences or individual bait constancy was observed. GC-MS analyses of 153 males of three species showed that individual hind tibiae contain highly variable quantities of a complex and species-specific blend of fragrance compounds, mainly terpenoids and aromatics. In all three species, frequency distributions of individual quantities were strongly skewed towards individuals with small amounts, and individual amount and complexity were positively correlated. Tibial contents of male *Euglossa imperialis* that were kept alive in a flight cage for 0, 5, 10, or 15 days showed no qualitative or quantitative change over time, suggesting that the fragrances are very efficiently stored in the hind legs. In *Euglossa cognata* wing wear, an established age correlate of the species, was positively correlated with individual fragrance quantity. Our results suggest that male euglossines forage continuously for a variety of volatiles, store them, and finally acquire large quantities of a complex and specific fragrance bouquet. Both qualitative and quantitative aspects of individual contents are likely to contain information on male phenotypic and genotypic quality.

Key Words—Euglossine bees, fragrance collection, sexual selection, species recognition, GC-MS.

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INTRODUCTION

The males of all neotropical orchid bees (Apidae: Euglossini) share the peculiar characteristic of collecting exogenous volatile substances (fragrances) from varied floral and nonfloral sources (Dressler, 1982). The fragrances are absorbed with modified foretarsal brushes and transferred into spongelike, hair-filled cavities in the grossly enlarged male hind tibiae (Vogel, 1963, 1966; Kimsey, 1984). During fragrance collection, the males also apply a mixture of certain nonpolar labial gland lipids to the fragrant surface. This seems to help them to retain the volatiles and therefore increases the efficiency of fragrance uptake, analogous to enfleurage, a process of scent extraction widely used by the perfume industry (Whitten et al., 1989). It is still not known what the males do with the collected fragrances, and the ultimate causes of the phenomenon are obscure. One view is that male bees accumulate large quantities and varieties of fragrances over time and that the individual fragrance bouquet serves as an indicator of male genotypic and phenotypic quality (e.g., reflecting foraging skills and/or survival abilities) to female bees (Kimsey, 1982; Whitten et al., 1989). If females prefer males with certain fragrance phenotypes, fragrance collecting behavior could have evolved by sexual selection. So far no direct evidence exists to support this hypothesis.

Males of some species of orchid bees have been observed to establish small mating territories in the vicinity of treefalls in dense forest. Within these territories the males perch and display on vertical tree trunks (Dodson, 1966; Kimsey, 1980; Stern, 1991). Matings are known to take place in these territories (Dodson, 1966; Kimsey, 1980), but so far it is unclear whether the fragrances play a role in courtship behavior or, more generally, whether they are exposed at all during male display.

As has been known for some time, male euglossines can be attracted to a number of pure artificial fragrance compounds that are presented in the bees' natural habitat (Dodson et al., 1969). In an extensive one-year study on Barro Colorado Island (BCI), Panama, Ackerman (1989) found that the males of a given species were attracted to up to 10 of the 16 compounds that were exposed. There was considerable overlap in fragrance choice between species, but each showed attraction to a characteristic set of compounds.

We studied qualitative and quantitative aspects of fragrance collection and storage of male orchid bees in the Barro Colorado Natural Monument (BCNM) in lowland Panama. To evaluate the potential for sexual selection in promoting male fragrance collecting behavior, we were particularly interested in the temporal patterns of fragrance accumulation and asked whether fragrance quality or quantity are likely to reflect individual foraging history and survival of male bees. Using various methods we attempted to answer the following questions:

1. Do male Euglossini forage for fragrances over long periods of their lifetime?

2. Are individual males interested in collecting a variety of compounds as expected from observations at the species level (Ackerman, 1989)?

3. What quantities and qualities of fragrances are found in the hind tibiae of individual males captured at fragrance baits?

4. What happens to the fragrances once they have been transferred into the hind tibiae? Are males able to retain the volatiles over time, or are the fragrances lost, chemically modified or exposed during display?

5. Is there a relationship between the age of individual bees and the quantity of fragrances in their hind tibiae?

METHODS AND MATERIALS

Mark–Recapture at Artificial Fragrance Baits. To assess individual fragrance choice and the temporal patterns of fragrance collecting behavior, we individually marked and released male euglossines captured at artificial fragrance baits on BCI. A total of eight compounds was used: 1,8-cineole (c), benzyl acetate (ba), methyl salicylate (ms), *p*-dimethoxybenzene (p-db), vanillin (v), *p*-cresyl acetate (p-ca), terpinene-4ol (t-ol), and *t*-methyl cinnamate (t-mc). These compounds were previously known to be good attractants for a range of euglossine bee species (Ackerman, 1989; Whitten, unpublished observations). Fragrances were simultaneously exposed on herbarium blotter pads in the radio tower clearing on BCI on 25 days (for 2–4 hr between 9:00 and 14:00 hr) between May 31 and July 23 in 1994 (every second day, except on rainy days). The baits were concealed in a screen of fine nylon mesh and not directly accessible to the bees. However, attracted bees brushed extensively on the mesh as if they were collecting fragrances. Males of two species [*Euglossa cognata* (small species) and *Eulaema meriana* (large)] were caught with hand nets and marked individually with colored numbered bee tags that were glued to the thorax with Super Glue. A total of 321 *Eg. cognata* and 120 *El. meriana* were marked. In case of repeated visits to the baits, the markings could normally be read without disturbing the bees. For each visit of marked bees, we recorded the bait, date, and time. To obtain information on the quality of wing wear as an age correlate, we repeatedly captured and remeasured a subset of the marked *Eg. cognata*. As individuals showed only marginal wing damage, we refrained from establishing damage classes based on area (as, e.g., Stern, 1991; Mueller and Wolf-Mueller, 1993) but rather measured wing wear as the number of nicks in fore- and hind wings on both sides of the body. Readings were made using a standard hand lens (magnification: 9×).

Fragrance Analysis and Samples. We analyzed the tibial contents of a total of 153 individual orchid bees belonging to three species of the genus *Euglossa* using gas chromatography-mass spectrometry (GC-MS). The bees were cap-

tured at concealed fragrance baits (see above) in the Barro Colorado Natural Monument (BCNM) between June 27 and July 27 in 1994. If not stated otherwise below, the individuals were placed in Eppendorf caps, cooled to immobility using a hand-held cooler (ice chest), and transferred to the laboratory within 2 hr following capture. There we immediately removed both hind legs of the individuals and placed them pairwise in glass vials (1 ml crimp-top vials with viton or butyl septum) that were filled with 0.5 ml *n*-hexane. The *n*-hexane contained 1 mg/liter 2-undecanone as internal standard (ISTD). GC-MS analysis was done using a Hewlett-Packard 5995 GC-MS with a 30-m DB-5 column at the Department of Natural Sciences at Florida Museum of Natural History. The oven temperature was programmed from 25 to 290°C at 3°C/min. Most fragrance compounds were identified by comparison with authentic standards, and spectra of unidentified compounds were searched against the NIST/EPA/NIH spectral library (Ausloos et al., 1992). When exact identification proved impossible, compounds were classified as fragrance compounds if they were of clear terpenoid or aromatic character (based on mass spectra). Thus, in almost all cases a clear separation between fragrances and labial gland lipids (mostly acetates and diacetates) was possible (see Whitten et al., 1989). The following samples were analyzed: 54 *Euglossa cognata*, captured at *p*-dimethoxybenzene, methyl salicylate, and vanillin baits [41 captured on Barro Colorado Island (BCI), 13 at the nearby mainland (Pipeline Road)]; 50 *Euglossa tridentata*, captured at cineole and vanillin baits on BCI; and 49 *Euglossa imperialis*, captured at cineole baits on BCI, subjects of the fragrance withdrawal experiment described below.

Data Analysis. Retention times and standardized peak areas (integrated ion current, measure of quantity) of identified fragrance compounds were recorded. Total individual amounts of fragrances were calculated by summing peak areas of all compounds present in a sample. Thus, within- and between-species comparisons of individual fragrance quantities were possible. To assess intra- versus interspecific variation of individual fragrance composition (quality), we calculated cluster analyses using NTSYS 1.8 (Rohlf, 1993). For that purpose a subset of individuals of the three species was selected that were similar in terms of total fragrance amount and complexity. A minimum of seven identified compounds and an upper limit of total fragrance amount of 15×10^6 units of total integrated ion current served as numeric criteria. The quantitative fragrance data of the subset were then transformed into qualitative presence/absence data. The resulting 0/1 matrix served as the basis for calculating distances using the Simple Matching Coefficient and Dice Coefficient in the SIMQUAL module of NTSYS 1.8. Clustering was done with UPGMA in the SAHN module.

Fragrance Withdrawal Experiment. To test for an effect of time on quantity and quality of individual fragrance contents, we conducted a cage experiment on *Euglossa imperialis*, a common euglossine bee in Central Panama. Forty-nine males of this species were captured at concealed cineole baits. Fifteen of these

were killed at once and their hind legs were sampled for GC-MS as described above. The other individuals were kept alive in a large (7 × 3.5 × 2 m) nylon mesh flight cage that was set up in the BCI laboratory clearing. During the first days of captivity, the bees learned to feed at artificial flowers (Eppendorf tubes with a yellow plastic corolla) holding a honey–water solution (50%) that was renewed daily. No obvious fragrance sources of any kind were present in the cage, nor were any males ever observed to collect exogenous substances during their captivity. On day 5 of the experiment some males started to perch and display on several of the 15 dead vertical tree tunks (3–6 cm in diameter) present in the cage. The observed behavior was found to be very similar to the territorial behavior of *Eg. imperialis* in its natural habitat as described by Kimsey (1980). Territorial activity in the cage continued to increase towards the end of the experiment until a substantial (but unknown) fraction of the males present were engaged in this behavior. After 5, 10, and 15 days of captivity random samples of 10, 10, and 12 individuals were captured and their hind legs were extracted in *n*-hexane. GC-MS analysis and data processing was carried out as described above.

RESULTS

Mark–Recapture. Table 1 summarizes the results of the study. The proportion of resighted individuals was high (around 20%) in both species of euglossine bees. Some individuals visited the baits repeatedly (up to three times in *El. meriana* and up to six times in *Eg. cognata*), partly covering substantial periods of time (up to a maximum of 44 days in both species; Figure 1). Resighted individuals of *Eg. cognata* were more likely to be resighted again than was expected from the average probability of resightings in that species ($\chi^2 = 16.2$; $N = 386$; $P < 0.001$). This is reflected by the fact that 84 of the 120 resighting events were due to only 29 individuals that were resighted repeatedly (more than once).

Male bees of both species did not exhibit obvious individual preferences for certain bait chemicals but switched between baits in a way that seemed to reflect the fragrance preferences that are characteristic of the species as a whole. Individual bees visited up to three different bait chemicals. In *Eg. cognata*, for which we had a sufficient sample size (number of resightings), we were able to test for individual bait constancy. Of 59 males that were first sighted at *p*-dimethoxybenzene (p-db) baits, 53 were resighted at p-db and six at methyl salicylate baits. These frequencies do not significantly differ from the frequencies found for first visits at the species level ($\chi^2 = 2.07$; $N = 376$; NS).

Individual Fragrance Contents—General Remarks. Only individuals of the three species that were collected at fragrance baits on Barro Colorado Island were included in the analysis. Besides *Euglossa cognata* ($N = 41$) and *Euglossa triden-*

TABLE 1. SUMMARY OF RESULTS OF MARK–RECAPTURE STUDY AT ARTIFICAL FRAGRANCE BAITS ON BARRO COLORADO ISLAND

	Marked individuals (*N*)	Compound visited when captured for first time (%)	Resightings (*N*)	Number of Resighted individuals [N(%)]	Resightings/ individual [N, mean (range)]	Time between first and last sighting [days, mean (range)]	Compounds visited/ resighted ind. [N, mean (range)]
Euglossa cognata	321	p-db: 80 ms: 18.7 v: 1.3	120	65 (20.2)	1.85 (1–6)	8.98 (0–44)	1.3 (1–3)
Eulaema meriana	120	ms: 47.5 c: 20.1 ba: 16.7 t-ol: 5.8 p-ca: 5.8 v: 0.8	37	24 (20.0)	1.5 (1–3)	12.0 (0–44)	1.6 (1–2)

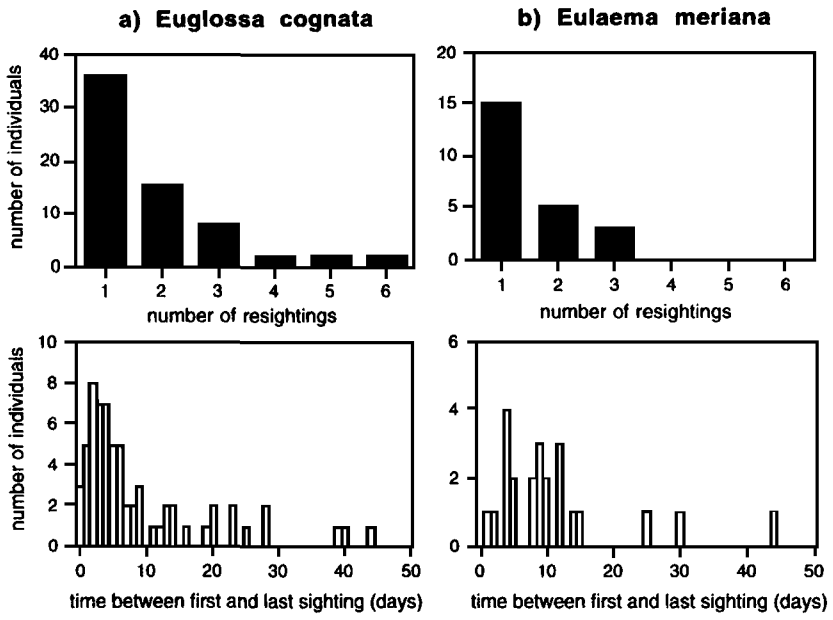

FIG. 1. Frequency distributions of the number of resightings/individual (above) and of the time between first and lasting sighting (below) of *Euglossa cognata* (a) and *Eulaema meriana* (b). Only individuals that were resighted at least once are included.

tata (N = 50), we also decided to include the 49 individuals of *Euglossa imperialis* that were subject to the fragrance-withdrawal experiment. The inclusion is based on the assumption that fragrance quantity and quality did not change over time during their captivity. This is confirmed by the results of the experiment (see below); therefore, a comparison with the two other species seems justified. Hind tibial extracts of individual males contained variable quantities of partly complex mixtures of fragrance compounds, consisting of open monoterpenes, bicyclic monoterpenes, sesquiterpenes, and diverse aromatics. A total of 143 different fragrance compounds were found in the three species. Retention times and, if available, identifications of the compounds as well as their distribution among species are given in the Appendix.

Quantity and Complexity of Individual Fragrance Contents. Table 2 summarizes the results of the GC-MS analysis for the three species. Quantity and complexity of the fragrance contents varied considerably between species and individuals. On average individuals of *Euglossa cognata* contained the lowest quantities, surpassed by *Euglossa imperialis* (–2× cognata) and finally *Euglossa tridentata* (–5× cognata). The average complexity of the individual contents as

TABLE 2. SUMMARY OF GC-MS ANALYSES OF HIND TIBIAE OF 141 INDIVIDUAL MALES OF THREE SPECIES OF *Euglossa* FROM BCI[a]

	Eg. cognata (N = 41)	*Eg. imperialis* (N = 49)	*Eg. tridentata* (N = 50)
Fragrance quantity			
Median ($\times 10^{-6}$)	3.52	6.37	17.7
Quartiles ($\times 10^{-6}$)	2.36/5.95	2.71/9.84	6.54/38.50
Skewness of distribution	2.03	1.59	1.57
Fragrance complexity			
Total number of fragrance compounds	34	51	105
Median of number of compounds/individual	6	8	16
Quartiles	4/7	4/12	10/28

[a]Values of fragrance quantity are sums of integrated ion currents $\times 10^{-6}$.

well as the total number of compounds found per species followed the same pattern.

A large amount of variation, however, was also found among individuals within species. Figure 2a shows the frequency distributions of individual fragrance quantities. In all three species the distribution is highly skewed towards individuals with comparatively small amounts of fragrances. Few males had large fragrance stores. Although emphasis must be given to between species differences (note different scales on the x axes), this general pattern was remarkably similar among species. In all three species there was a strong positive correlation between individual fragrance quantity and complexity (Figure 2b). Individual mixtures extracted consisted of up to 47 different fragrance compounds.

Composition and Specificity of Individual Fragrance Contents. As emphasized by the data given in the Appendix, we found considerable overlap in the fragrance composition among species. Of the 143 compounds found, 12 were present in all three species and another 23 occurred in two of the three species. However, in spite of this partial overlap, cluster analyses revealed greater similarity of fragrance composition within than between species (Figure 3). Both similarity indices used in combination with UPGMA proved to be consistent and produced exclusively species specific clusters. The observed differentiation between species was mainly caused by differences in major compounds that were present in a large fraction of the individuals of a given species (see Appendix).

Fragrance Storage Over Time. Figure 4 shows the medians and quartile ranges of individual fragrance quantities of 47 male *Euglossa imperialis* grouped by the duration of their captivity. Statistical analysis did not reveal any decrease

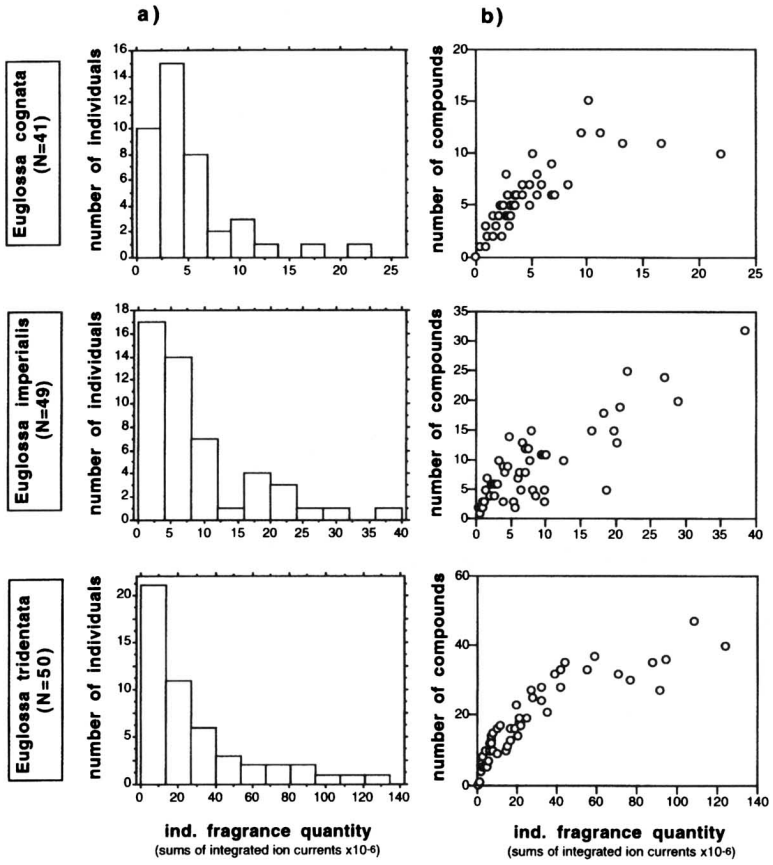

FIG. 2. (a) Frequency distributions of individual fragrance quantities of three species of *Euglossa* on BCI revealing considerable variation and high levels of skewness towards individuals with only little amounts. (b) Relationship between individual fragrance quantity and complexity (number of fragrance compounds).

or increase in the individual amounts of fragrances over time (Kruskall-Wallis ANOVA: $H = 2.50$; $N = 47$; $df = 3$; $P > 0.48$). To test for changes in fragrance quality, we selected the eight major compounds found in the *imperialis* extracts (hexahydrofarnesyl acetone, cineole, methyl salicylate, α-pinene, nerolidol, sabinene, germacren D, and β-pinene) and, for each compound, tested for changes over time using Kruskal-Wallis ANOVA. Two of the compounds (nerolidol and germacren D) significantly increased ($H = 14.06$, $P < 0.01$; and $H = 10.30$; $P < 0.05$), whereas the other compounds exhibited no change over time.

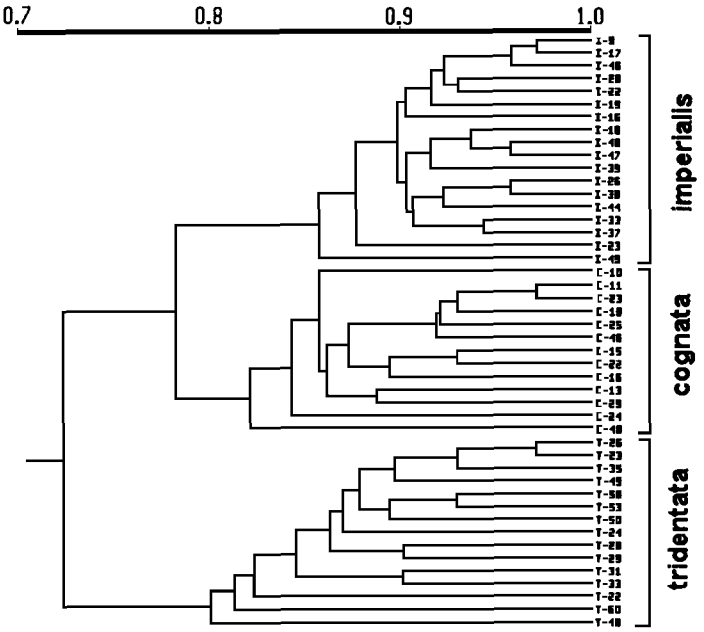

FIG. 3. Dendrogram of individual males of three sympatric species of *Euglossa* from BCI, based on qualitative similarity of fragrance composition extracted from the hind tibiae. Distances are simple matching coefficient, clustering was done with UPGMA in NTSYS 1.80 (Rohlf, 1993). Individuals cluster in three groups according to their species.

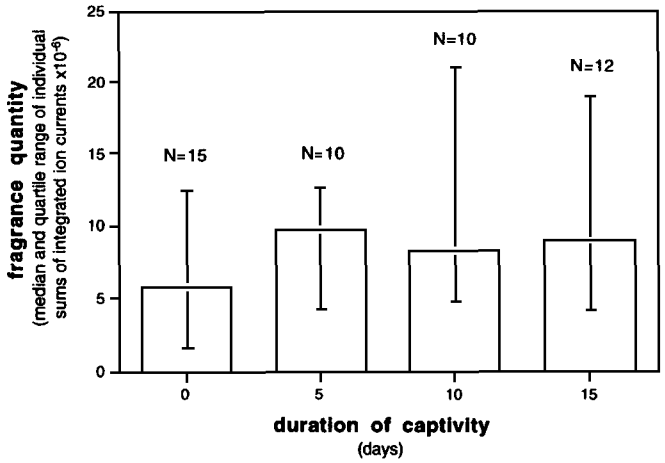

FIG. 4. Median and quartile range of the fragrance quantities of male *Euglossa imperialis* plotted against the duration of their captivity. No change over time was observed.

Fragrance Quantity and Individual Age. We tested the applicability of wing wear, measured as the number of notches in the edges of fore- and hind wings, as an age predictor for male *Eg. cognata* during the mark–recapture study (see above). The increase in wing wear of individual males was dependent on time between first and last measurement at the fragrance baits (Regression analysis; $y = 0.423x - 0.609$; $r^2 = 0.76$; $F = 105.92$; $N = 35$; $df = 1$; $P < 0.001$; Figure 5a). However, individual rates of accumulation showed considerable variation,

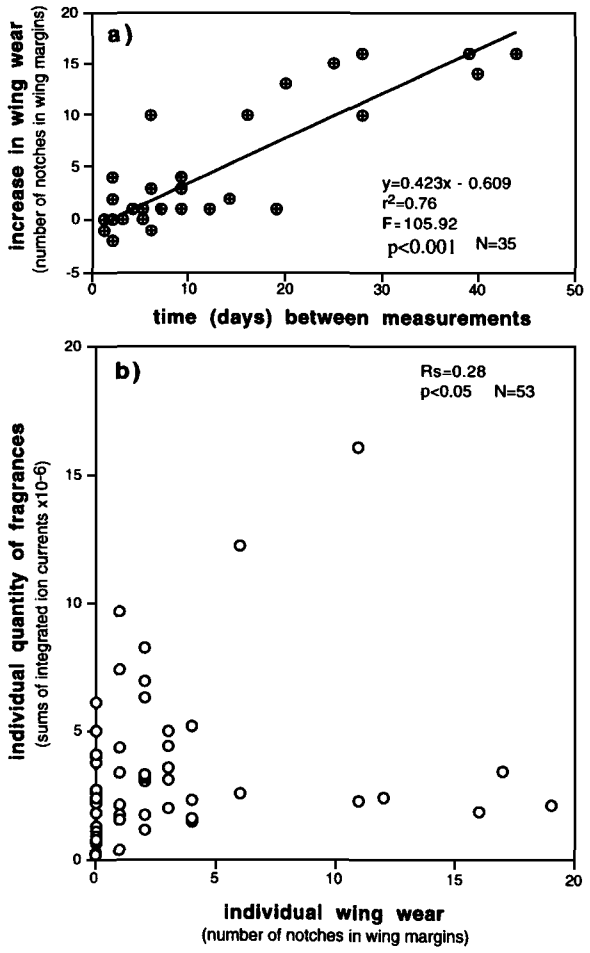

FIG. 5. (a) Dependency of the amount of increase of wing wear on the time between measurements in *Euglossa cognata.* (b) Correlation between the individual quantity of fragrances stored and individual wing wear.

limiting the value of wing damage as a precise age indicator. Nonetheless, there was a weak but significant positive correlation between individual wing wear and the amount of fragrances stored by male *Eg. cognata* (Spearman $R_s = 0.28$; $N = 53$; $p < 0.05$; Figure 5b).

DISCUSSION

Fragrance Quality and Specificity. The results of our GC-MS analyses showed that the hind tibiae of individual males contain variable quantities of complex blends of terpenoid and aromatic fragrance compounds. Male euglossines have been observed collecting volatiles from a variety of sources including flowers of Orchidaceae, Solanaceae, Bignoniaceae, and Euphorbiaceae, as well as nonfloral sources such as decaying wood, rotting fruits, feces, and tree wounds (Zucchi et al., 1969; Dressler, 1982; Williams, 1982; Whitten et al., 1993). The scents produced by attractive sources, if analyzed, vary considerably in their complexity and chemical composition. The bouquets of euglossophilous orchids typically consist of 3–10 different compounds, of which one or two are dominant (Williams and Whitten, 1983; Gerlach and Schill, 1991, Kaiser, 1993), whereas scents of other plant species visited by euglossines can be even more complex (e.g., Sazima et al., 1993). Pure single-compound scents may be rare in the bees' natural habitat, but have been shown to occur in decaying wood visited by euglossines (Whitten et al., 1993; see below). The majority of the identified compounds found in our *Euglossa* samples are known from floral and/or nonfloral sources, but, due to the seemingly endless number of potential combinations, it is presently impossible to link certain sets of compounds to specific fragrance sources.

The tibial fragrance contents of each respective *Euglossa* species were dominated by a certain number of major compounds present in the majority of the individuals. Apart from these, the extracts contained a varying number of minor compounds (usually in low quantities) only found in a small number of individuals. One might conclude that it is the major compounds that the bees are actually seeking, whereas the minor ones are unintentionally accumulated. However, the truth is likely to be more complicated, as some of the minor components prove to be highly attractive to the respective species when exposed in pure form as fragrance bait. Vanillin, e.g., was present in the extracts of only three individuals of *Eg. tridentata*, but is known to be one of the strongest attractants of this species in Central Panama (Ackerman, 1989; T. Eltz, unpublished observation). Generally, there is no simple relationship, positive or negative, between the predominance of certain compounds in a species' extracts and their attractivity in bioassay. Of the two most dominant compounds found in samples of *Euglossa imperialis*, one, cineole, is its strongest known attractant (e.g., Ack-

erman, 1989), whereas the other, hexahydrofarnesyl acetone, completely failed to attract any bees (T. Eltz, unpublished observation). The reasons for this are entirely unknown. Many fragrance compounds are chiral, and bees might be attracted only to certain enantiomers and neglect others. In addition, some of the fragrance compounds may only be perceptible to the bees when present in combination with modifying components. Detailed studies of male chemoreception in combination with adequate bioassays are required to resolve this issue.

In spite of considerable intraspecific variation in fragrance composition, the results of our cluster analyses show that the bouquets of the three *Euglossa* species are sufficiently different from each other to allow the individuals to be assigned to their respective species. Thus, we have demonstrated for the first time that the fragrances of male euglossines have the potential of being used as a means of species recognition. Whether they are actually used in that process remains an open question.

Fragrance Accumulation. During our mark–recapture study, several males of both species periodically visited the artificial fragrance baits over considerable periods of time (up to 44 days), suggesting that the bees do engage in some fragrance foraging during most, if not all, of their lifetime. The findings certainly underestimate the true extent of time and energy allocated to the collection of volatiles, as the marked bees most likely have visited natural fragrance sources as well. So far no information has been published on the quantity of volatiles that a collecting bee can extract from natural fragrance sources. Preliminary data from a study on Cerro Campana in Central Panama suggests that quantities can be small indeed. Tibial extracts of 15 male *Euglossa deceptrix* that had collected fragrances at a decaying log for 1–10 min did not contain any detectable traces of the single volatile compound produced by the log (*p*-dimethoxybenzene) (Eltz and Whitten, unpublished data). Although it is unknown whether the log from Cerro Campana can be considered an ordinary euglossine fragrance source, it is likely that fragrances are generally scarce and that the bees must allocate considerable time and effort to the acquisition of large fragrance quantities.

Although the evidence is circumstantial, our results do suggest that the males accumulate the fragrances continuously over considerable periods of time, perhaps throughout their lifetimes. The following findings support this hypothesis of long-term fragrance accumulation:

1. The fragrances can be stored very efficiently in the hind tibiae over long periods of time, as evidenced by the absence of any change in fragrance quantity during the fragrance-withdrawal experiment. This superior storage capability is probably achieved through the combined effects of the retaining properties of the nonpolar carrier lipids secreted from the labial glands (Whitten et al., 1989) and the strong capillary forces imposed by the sponge-like morphology of the invaginated cuticle inside the hind tibia that serves as a storage container (see Vogel, 1963, 1966; Cruz-Landim et al., 1965).

2. There is a positive relationship between the individual fragrance quantity and the established age correlate wing wear in *Euglossa cognata*. The weakness of the relationship may partly result from the low accuracy of wing wear as an absolute age predictor (variable individual rates of acquiring wing wear), as well as from the influence of confounding variables that lead to individual differences in the rate of fragrance acquisition (e.g., foraging skills and activity, see below).

3. The frequency distributions of the individual fragrance quantities are skewed towards individuals with only small amounts in all three species. Assuming long-term fragrance accumulation, such a distribution would be expected from a population that is numerically dominated by young individuals. Wing wear data from baiting studies in Panama suggest that euglossine populations do indeed frequently show age distributions skewed towards young individuals (Ackerman, 1983; Zimmerman and Madrinan, 1988; D. W. Roubik, unpublished data).

4. In all three species there was a strong positive correlation between quantity and complexity of individual fragrance stores. Although this relationship may be partly an artifact of the GC-MS analysis (more small peaks may remain underneath the detection threshold in low-quantity extracts), there is certainly some biological substance to it. During foraging from diverse and ephemeral fragrance sources, the males are likely to add new compounds continuously to their acquired fragrance bouquets. Thus, a correlated increase in quantity and complexity is in general agreement with the hypothesis of long-term fragrance accumulation.

Any evaluation of the hypothesis is of course hampered by the complete lack of indications on the actual use of the fragrances by the males. The results of the fragrance-withdrawal experiment suggest that the volatiles are neither absorbed into the hemolymph for further processing and translocation to exocrine organs as hypothesized by Williams (1982), nor chemically modified within the tibial cavities. Furthermore, as fragrance quantities remained unaltered over time, there was no indication that the bees actually exposed substantial amounts of the fragrances during territorial display, as was suggested by Vogel (1966). However, it can hardly be doubted that the fragrances are used at some point in the males' lives. At present, the most likely scenario is that the volatiles are only exposed during the brief and rarely observed encounters of courtship between the sexes at the males' territories.

If the hypothesis of long-term fragrance accumulation is correct, then the accumulated bouquet of males will contain information on the individuals' foraging history and survival. This intrinsic information on male performance may be the basic prerequisite for sexual selection to act on and shape male fragrance collecting behavior. If the ability of individual males to find and exploit fragrance sources and to survive at the same time, varies, and if this variation has a genetic background, the fragrance bouquet will in some way reflect male genotypic qual-

ity. If females have inherited preferences for certain high-quality fragrance phenotypes, then fragrance collection could have evolved through female choice and sexual selection. The collection of fragrances most probably does inflict the high, survival-reducing costs on male bees that are required by genetic handicap or indicator models (Zahavi, 1975, 1977; Maynard Smith, 1985; Pomiankowsky, 1987), e.g., an increased risk of predation or the costly detoxification of certain toxic fragrance compounds. In addition to indirect genetic benefits (high-quality offspring), female euglossines may also gain direct benefit from choosiness. Female preference for males that closely match a specific optimum fragrance phenotype may reduce the risk of wasteful and potentially fatal hybrid matings. As outlined above, individual fragrance bouquets do indeed provide clues for species recognition.

So far it is unclear what exact aspect of the individual fragrance bouquet (quantity, complexity, specificity, or a combination of these) is the true target of selection. Detailed studies on the sexual behavior and sensory physiology of orchid bees are required to obtain further insights into the evolution of fragrance collection.

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APPENDIX. FRAGRANCE COMPOUNDS FOUND IN HIND TIBIAE OF THREE SPECIES OF *EUGLOSSA* FROM BCI[a]

RT (min)	Compound	*Eg. cognata* (N = 41)	*Eg. imperialis* (N = 49)	*Eg. tridentata* (N = 50)
9.25	α-thujene		6	
9.48	α-pinene	2	**31**	16
9.72	benzaldehyde			12
10.38	camphene		6	
10.80	sabinene		**27**	9
10.93	β-pinene	1	**25**	11
11.20	myrcene	1	14	19
11.81	*p*-menth-2-*ene*, trans		1	
11.92	α-phellandrene		9	4
12.22	3-carene		13	3
12.40	α-terpinene		3	
12.68	*p*-cymene		1	
12.78	limonene	4	5	4
12.80	β-phellandrene		2	
12.95	cineole		**42**	**37**
13.18	(*Z*)-ocimene	1	4	11
13.88	(*E*)-ocimene		1	**29**

APPENDIX. CONTINUED

RT (min)	Compound	*Eg. cognata* (*N* = 41)	*Eg. imperialis* (*N* = 49)	*Eg. tridentata* (*N* = 50)
14.08	γ-terpinene		4	
	?		4	
15.12	terpinolene	4	6	4
	?		2	
15.47	linalool	1	4	2
16.03	phenylethyl alcohol			4
16.85	2,6-dimethyl-2,4,6-octatriene			3
17.50	ipsdienol	11		13
18.25	*p*-dimethoxybenzene	**23**		21
18.68	benzoic acid			8
18.78	menthol		1	
18.98	terpinen-4-ol		2	
19.58	α-terpineol		4	2
19.73	methyl salicylate	10	**32**	2
20.95	2,3-dihydrobenzofuran			13
22.38	*p*-anisaldehyde			1
22.58	phenylethyl acetate			1
23.67	anis alcohol isomer			1
23.93	anis alcohol			2
23.97	anethole			4
24.88	*trans*-cinnamyl alcohol			1
26.23	?			19
27.20	eugenol			**37**
27.78	1,2,4-trimethoxybenzene			1
27.87	decanoic acid			8
28.28	α-copaene		8	1
28.35	*trans*-methyl cinnamate			6
28.42	geranyl acetate			2
28.85	β-cubebene		3	
28.93	vanillin			4
28.93	germacrene A	4	4	23
29.27	methyl eugenol			1
29.88	anisyl acetate			1
30.10	?	9		
30.17	caryophyllene	2	18	9
30.52	cinnamic acid	1		
30.53	?	2		
30.77	?		2	1
30.82	(*Z*)-*trans*-α-bergamotene	10		1
30.82	paeonol		1	
31.15	?			1
31.18	*trans*-isoeugenol			4
31.22	?			1
31.38	geranyl acetone		1	

APPENDIX. CONTINUED

RT (min)	Compound	*Eg. cognata* (*N* = 41)	*Eg. imperialis* (*N* = 49)	*Eg. tridentata* (*N* = 50)
31.60	*cis*-β-farnesene	**28**		5
31.66	α-humulene		4	1
31.83	?			18
31.90	ethyl cinnamate			1
32.00	NIST 27977		1	
32.60	β-himanchalene ?	1		
32.65	γ-muurolene		2	
32.82	β-cubebene		1	
32.87	germacrene D	**33**	**27**	6
33.23	bergamotene?	1		
33.25	α-zingiberene	1		
33.27	*trans*-methyl isoeugenol		1	
	α-selinene		1	
33.50	germacrene B?	2		
33.50	α-selinene			1
33.62	?			5
33.85	(*E*, *E*)-α-farnesene	**24**	7	1
33.68	β-bisabolene			**35**
34.23	γ-cadinene		6	
34.52	δ-cadinene		6	
34.53	*trans*-β-farnesene	3		9
34.83	?			17
35.03	?			**29**
35.03	?			1
35.28	?			1
35.52	?			18
35.58	?			2
35.67	elemicin	7		1
36.02	nerolidol	**22**	**31**	4
36.08	dodecanoic acid			5
36.15	methoxycinamaldehyde?			**30**
36.22	?			**29**
36.73	?			1
36.85	?			1
37.03	cis-methyl *p*-methoxycinnamate			4
37.55	methoxyeugenol	1		3
37.68	farnesene oxide I	1		
38.23	farnesene oxide?		1	9
38.27	?		2	3
38.35	farnesene oxide II	16		
39.27	zingiberone		8	
39.38	isoelemicin	13		
39.67	NIST 11930		1	

APPENDIX. CONTINUED

RT (min)	Compound	*Eg. cognata* (*N* = 41)	*Eg. imperialis* (*N* = 49)	*Eg. tridentata* (*N* = 50)
39.87	?	1		
40.25	trans-methyl-*p*-methoxycinnamate			8
40.55	elemol?			6
40.58	?			**34**
40.62	?			1
40.90	α-bisabolol	1		11
41.37	?			20
41.73	?			11
41.85	?	2		
42.12	spathulenol?		12	
42.17	farnesol?			2
43.77	benzyl benzoate			**38**
45.57	?			3
45.93	hexahydro farnesyl alcohol?		1	4
46.55	hexahydrofarnesyl acetone		**44**	2
47.28	benzyl salicylate			5
50.13	decanoic acid, phenylmethyl ester			3
51.37	?			2
51.88	aff. hexahydrofarnesyl alcohol		4	
52.57	geranyllinalool			2
53.45	?			7
54.15	?			**36**
54.58	benzyl cinnamate	1		10
56.00	?			2
56.02	dodecanoic acid, phenylmethyl ester			1
57.53	?			**40**
58.43	?			2
58.95	?			18
59.97	?			**33**
60.18	?			6
60.42	?			3
62.10	?			2
62.58	?			6
65.35	?			14
67.05	?			1
68.93	?			8
70.17	?			4
70.45	?			1

[a](*Eg. cognata*, *N* = 41; *Eg. imperialis*, *N* = 49; *Eg. tridentata*, *N* = 50). Counts refer to the number individuals that contained the respective compound. Counts of dominant compounds (found in more than 50% of individuals of a given species) are given in bold numerals.

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COMPARATIVE STUDY OF PHEROMONE PRODUCTION AND RESPONSE IN SWEDISH AND ZIMBABWEAN POPULATIONS OF TURNIP MOTH, *Agrotis segetum*

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Abstract—Analysis of female sex pheromone gland extracts of the turnip moth (or common cutworm), *Agrotis segetum*, from Zimbabwe revealed three compounds previously identified as sex pheromone components in the Swedish population, namely (*Z*)-5-decenyl acetate (Z5–10 : OAc), (*Z*)-7-dodecenyl acetate (Z7–12 : OAc), and (*Z*)-9-tetradecenyl acetate (Z9–14 : OAc). However, the proportions from the Zimbabwean population (1 : 0.25 : 0.03) differ from those in the Swedish population (1 : 5 : 2.5). In addition, gas chromatography–mass spectrometric (GC-MS) analysis of the Zimbabwean female gland extracts revealed a trace of (*Z*)-5-dodecenyl acetate (Z5–12 : OAc). This compound has recently been identified as a fourth sex pheromone component for the Swedish population. Single-sensillum recordings from both Zimbabwean and Swedish populations showed the presence of two types of antennal receptors responding to either Z5–10 : OAc or Z7–12 : OAc. In Zimbabwean males the Z7–12 : OAc receptor neuron appeared to be confined to the basal and medial thirds of the antennal branches, while in Swedish males it was distributed along the entire antennal branch. Dose–response curves of Z5–10 : OAc or Z7–12 : OAc specific receptor neurons from males of both populations showed similar response profiles, but the neurons of the Zimbabwean population showed higher maximal responses. In flight tunnel tests with Zimbabwean males, the three-component Zimbabwean blend of Z5–10 : OAc, Z7–12 : OAc and Z9–14 : OAc elicited significantly greater responses than the Swedish blend, but not significantly greater than pheromone glands from calling Zimbabwean females. (*Z*)-5-decenol (Z5–10 : OH), a constituent of gland extracts, exerted an antagonistic effect

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in the flight tunnel. In field tests conducted in Sweden, local males were preferentially attracted to local females, while in Zimbabwe preferential attraction to local females was less pronounced. Local response to the Swedish and Zimbabwean synthetic four-component blends mirrored the responses to the local females. Zimbabwean males are much more strongly attracted to Z5–10 : OAc alone than are Swedish males and the high concentrations of Z7–12 : OAc and/or Z9–14 : OAc present in the Swedish blend reduced attraction of Zimbabwean males. This reduced attraction appears to be counteracted by the trace amounts of Z5–12 : OAc found in the Swedish four-component blend. Addition of Z5–12 : OAc to the three-component Zimbabwean blend did not, however, significantly increase the trap catches of Zimbabwean males.

Key Words—Sex pheromone, turnip moth, common cutworm, *Agrotis segetum*, geographical population variation, receptor neurons, single-sensillum recordings, dose–response, flight tunnel, field tests.

INTRODUCTION

The turnip moth (common cutworm of Africa), *Agrotis segetum* (Dennis and Schiffermüller) (Lepidoptera: Noctuidae), is a serious pest of many crops throughout Africa and most of Europe and Asia. Its sex pheromone has been previously identified as a blend of three components: (*Z*)-5-decenyl acetate (Z5–10 : OAc); (*Z*)-7-dodecenyl acetate (Z7–12 : OAc); and (*Z*)-9-tetradecenyl acetate (Z9–14 : OAc) (Bestmann et al., 1978; Arn et al., 1980; Tóth et al., 1980; Löfstedt et al., 1982). Recently another gland constituent, (*Z*)-5-dodecenyl acetate (Z5–12 : OAc) (Löfstedt et al., 1986), was demonstrated to be both electrophysiologically and behaviorally active (Wu et al., 1995), and thus qualifies as a fourth sex pheromone component.

Geographical variation in male responses to pheromone blends was first reported in field-trapping tests with several European populations of *A. segetum* (Arn et al., 1983). These tests showed that males from a French population were attracted not only to the ternary mixture but also to Z5–10 : OAc alone. Variation in the production of, and response to, different three-component blends was further demonstrated in the laboratory by analysis of female extracts as well as by investigations of olfactory sensilla on male antennae from several European populations. The results revealed a correlation between the proportions of the three components and the number of their corresponding olfactory receptor neurons (Löfstedt et al., 1986; Hansson et al., 1990).

A recent survey examined differences in the responses of male *A. segetum* to five different synthetic pheromone mixtures (one ternary, two binary and two single components) at 11 widely separated geographical locations in Europe, Asia, and Africa (Tóth et al., 1992). At all localities in Eurasia as well as at the single north African site (Egypt), and despite more or less continuous vari-

ation in local responses, the most attractive blend was always the ternary mixture (Z5–10 : OAc, Z7–12 : OAc, and Z9–14 : OAc, 1 : 1 : 8). In contrast, at two southern African sites (Zimbabwe and South Africa), Z5–10 : OAc alone was definitely the most attractive to local males. This suggested that these African populations had a markedly different pheromone system from Palaearctic populations.

In addition to the geographical differences in the *A. segetum* pheromone system mentioned above, differences have also been described between the lepidopteran pheromone systems of sympatric putative sibling species, e.g., *Diacrysia chrysitis* and *D. tutti* (Löfstedt et al., 1994), between strains of the same species, e.g., *Ostrinia nubilalis* (Klun and cooperators, 1975; Kochansky et al., 1975; Roelofs et al., 1985), as well as between geographically separate populations of other species, e.g., *Choristoneura rosaceana* (Thomson et al., 1991), *Zeiraphera diniana* (Guerin et al., 1984), and *Planotortrix excessana* (Foster et al., 1986, 1989).

The biological significance of geographical variation in the pheromone systems of populations of the same species is not understood, and the results of Tóth et al. (1992) prompted us to study the pheromone system of *A. segetum* from Zimbabwe in greater detail and to compare it with that of the Swedish population. Hybrids between the Swedish and the Zimbabwean *A. segetum* have been established in our lab. The hybrid blend was also tested in the present study. In the present paper we report results from analyses of pheromone gland extracts, electrophysiological recordings of sensilla on male antennae, and behavioral investigations in a flight tunnel and in the field.

METHODS AND MATERIALS

Insects. A Zimbabwean culture of *A. segetum* was established in the Lund laboratory from pupae originating from Kutsaga Research Station, Harare, Zimbabwe. A Swedish culture of *A. segetum* was established from collections from southern Sweden and Denmark and maintained in the laboratory for over three years. Larvae of both populations were reared on a semisynthetic diet (Seshu Reddy and Davis, 1978) and were kept at 25°C on a 16-hr light–8-hr-dark cycle. Pupae were sexed based on the morphological difference at the ventral side of the last abdominal segment between males and females. Male pupae and adults were kept at 20°C with 14-hr light–10-hr dark photoperiod, while female pupae and adults were kept in a separate chamber at 23°C with a 16-hr light–8-hr dark photoperiod. First- to third-generation male Zimbabwean *A. segetum* were used for the electrophysiological and behavioral tests.

Chemicals. Z5–10 : OAc, Z5–12 : OAc, Z7–12 : OAc, Z9–14 : OAc, and (Z)-5-decenol (Z5–10 : OH) were obtained from our laboratory stock, initially

purchased from the Institute for Pesticide Research, Wageningen, The Netherlands, and were >99% pure with respect to geometrical isomers as determined by gas chromatography. For a single-sensillum screening test, 10 μg of each of the above compounds, diluted in 10 μl hexane, was applied to a piece of filter paper (8 × 18 mm) that was inserted in a Pasteur pipet. For dose–response single-sensillum recordings, serial dilutions (10^{-3}–10^{2} μg) of Z5–10 : OAc, Z7–12 : OAc, or Z5–10 : OH in the 10 μl hexane were used. The synthetic pheromone blends used for behavioral assays (Table 1) were checked by gas chromatography to ensure the purity and the ratios between components. The ratios of components in the hybrid blend were determined from females obtained from a cross between the Swedish and Zimbabwean *A. segetum*. In field tests, blends were applied to rubber septa in 50 μl hexane to achieve the desired dosage. In flight tunnel assays, blends were applied to filter paper in 10 μl hexane to achieve the desired dosage. In addition to the synthetic blends, pheromone glands from calling females and gland extracts also were tested in the flight tunnel.

Analyses of Pheromone Gland Extracts. The terminal abdominal segments with the pheromone glands were dissected from 2- to 4-day-old females during the fourth to seventh hour of the scotophase. Glands were either used immediately for flight tunnel tests or extracted in hexane for 2 hr to be used later for chemical analyses and for flight tunnel assays (Zimbabwean females only). The female extracts were analyzed on a Hewlett Packard 5880A gas chromatograph equipped with a DB-Wax column (30 m × 0.25 mm ID, J&W Scientific, Folsom, California). Hydrogen was used as carrier gas. Samples were injected splitless. The injector temperature was 225°C and the split valve was opened 1 min after injection. The column temperature was maintained at 80°C for 2 min following the injection and then linearly increased to 230°C at a rate of 10°C/min. Gas chromatographic–mass spectrometric (GC-MS) analyses were performed by HP

TABLE 1. DEFINITIONS OF SYNTHETIC BLEND CODES (SBC)

	Proportions of components				
SBC	Z5–10:OAc	Z5–12:OAc	Z7–12:OAc	Z9–14:OAc	Blend names
1	1		5	2.5	Swedish three-component blend
2	1	0.1	5	2.5	Swedish four-component blend
3	1				
4	1	0.1			
5	1		0.25		Zimbabwean two-component blend
6	1		0.25	0.03	Zimbabwean three-component blend
7	1	0.1	0.25	0.03	Zimbabwean four-component blend
8	1		1.61	0.92	Hybrid blend

GC-MS with electron impact ionization (70 eV), equipped with a 5970b computer system, and interfaced with an HP 5890 GC. For the detection of pheromone components from female extracts, the GC-MS was operated in the selected ion monitoring mode. Selected ions were monitored in several groups and the groups were changed at preset times during the separation. These times were based on the retention times of the synthetic standards. The following diagnostic ions were chosen for the detection of pheromone compounds, Z5–10 : OAc, m/z 138.20; Z5/Z7–12 : OAc, m/z 166.20; Z9–14 : OAc, m/z 194.20.

Electrophysiological Recordings. Single-sensillum recordings were performed by using the tip-cutting technique (Kaissling, 1974; Van Der Pers and Den Otter, 1978). A male moth was secured in a plastic pipet, the tip of which had been cut to allow the head to protrude. The antennae were then fixed by dental wax and positioned to allow microelectrode access. A thin silver wire, serving as ground electrode, was inserted into the abdomen. A sensillum was cut by means of microscopic glass knives, and a recording electrode with a tip diameter of about 4 μm, filled with Beadle-Ephrussi Ringer was placed in contact with the cut surface of the sensillum. The antenna was continuously flushed with charcoal-filtered and moistened air delivered through a glass tube (ID 8 mm) at a speed of 0.5 m/sec, the outlet of which was about 10 mm from the antenna. The stimulus was injected into the airstream through an aperture (3 mm diameter) in the tube in a 0.5-sec puff by a stimulation device (Syntech, P.O. Box 1547, NL-1200 Hilversum, The Netherlands).

Flight Tunnel Tests. Flight tunnel experiments were performed in a 2.5-m-long × 0.9-m-wide × 0.9-m-high Plexiglas flight tunnel as described by Löfstedt and Herrebout (1988). The flight tunnel conditions used were 21–23°C, 40–60% relative humidity, 0.3 m/sec wind velocity, and 0.6 lux light intensity.

Two- to 3-day-old Zimbabwean male moths were transferred individually to 250-ml cylindrical screen cages in plastic cups before the initiation of the dark period and allowed to acclimate to the conditions in the tunnel room for 1 hr before they were tested 3–5 hr into the scotophase. Males were released individually into the plume from a cylindrical screen cage with the open end facing upwind. Six behavioral steps typically exhibited in the flight tunnel were recorded, taking flight (TF), orientation (Or), upwind flight 50 cm from the release cage in the plume (50 cm), upwind flight half the distance between the source and the release cage in the plume (HW), close approach within 10 cm to the source (190 cm), and source contact (SC). A male was considered to have contacted the source as soon as its antennae touched it. Males were tested once and then discarded. After testing, the release cages were heated at 200°C for 2 hr and the insect needles holding the filter paper dispensers were rinsed in acetone.

Field Tests. Field tests were conducted with Lund II sticky traps (Anderbrant et al., 1989) placed in sugar beet fields in southern Sweden or by using

commercial delta traps (PLA Insect Monitoring Systems, UK) in tobacco lands at Kutsaga Research Station in Zimbabwe. Red rubber septa (Thomas Scientific, USA) were used as dispensers. Three of the experiments were carried out in both Sweden and Zimbabwe and two others only in Zimbabwe. For convenience of description and reference, the different kinds of experiments are numbered and prefixed with "S" if performed in Sweden or "Z" if performed in Zimbabwe.

The first two experiments (S1/Z1 and S2/Z2) were pairwise comparisons in which two traps, placed approximately 1 m apart, were used at each replicate site, with sites at least 23 m from one another. Trapped moths were counted and removed each day, or every other day, and at these times, the traps were switched in position to minimize position effects. For S1 (eight replicates) and Z1 (six replicates), each paired trap was loaded with one, same-aged virgin female (one Swedish and one Zimbabwean female per pair of traps). For S2 (24 replicates) and Z2 (15 replicates), each pair of traps was loaded with a septum carrying either synthetic blend code 2 or 7 (SBC 2 or SBC 7) (Table 1). The difference in the number of males trapped by each member of a pair of traps up to the last night on which both females were alive (S1 and Z1) or over five nights (S2 and Z2) was used as the response variable for analysis by a paired-sample Student t test. S1 and S2 were carried out from June 23 to July 1, 1994; Z1 from October 26 to November 10, 1994; and Z2 from January 23 to February 10, 1995.

For the remaining experiments, more than two blends were tested at each replicate site. Traps at a site were approximately 25 m apart and arranged in a row perpendicular to the predominant wind direction. Sites were at least 60 m apart in Sweden or 100 m apart in Zimbabwe. When trapped males were removed, the traps at a site were moved one position in a standard direction. Experiments S3, Z3a, and Z3b compared six blends (SBCs 1, 2, 3, 5, 6, and 8). S3 was carried out from June 3 to 7, 1993; Z3a from October 11 to 30, 1993; and Z3b from November 7 to December 4, 1993. Five replicate sites were used for each experiment. Bait septa were renewed every five days. The response variate analyzed was the total catch for each blend at the replicate sites.

Experiment Z4 compared five blends (SBCs 2, 3, 4, 6 and 7) at five sites using one set of baits exposed for five days between February 28 and March 5, 1994. The total number of males caught by each blend over the five days was analyzed.

Experiment Z5 involved three blends. One Swedish blend, one Zimbabwean blend, and one Zimbabwean blend with Z5–12 : OAc each were tested at four dosages (0.03–30 μg in decadic steps). Dosage refers to the amount of Z5–10 : OAc. The four dosages were tested at three rotating sites by using five sets of septa, each exposed for five nights, to give a total trapping period of 25 nights between March 5 and April 1, 1994.

Fisher's exact test was performed in experiments S1, Z1, S2, and Z2. In experiments S3, Z3, S4, Z4, and Z5, analyses of variance of $\ln(x + 1)$ trans-

formed catches and multiple comparisons between means were performed, with ANOVA followed by Fisher protected least significant difference (FPLSD) test for data which gave significant overall F values. In experiment Z3, the chi-square test of homogeneity was used to assess whether the two experiments Z3a and Z3b represent samples from the same population.

RESULTS

Analyses of Pheromone Gland Extracts. Gas chromatographic analyses of female gland extracts from Zimbabwean and Swedish populations revealed three pheromone components: Z5–10 : OAc, Z7–12 : OAc, and Z9–14 : OAc in clearly different proportions (Figure 1). The average ratio (±SEM) of the three compounds in 13 batches of Zimbabwean female gland extracts (the total number of glands in 13 batches was 100) was $1 : 0.25 \pm 0.09 : 0.03 \pm 0.02$, whereas the average ratio in the Swedish females was $1 : 4.8 \pm 1.1 : 2.5 \pm 1.0$ from 20 extracts of one female gland each. GC-MS analyses on both Swedish and Zimbabwean female extracts revealed that at m/z 166.20 there was a trace peak with the same retention time as Z5–12 : OAc.

Electrophysiological Recordings. A total of 108 sensilla from antennae of male Zimbabwean *A. segetum* were examined by stimulation with Z5–10 : OAc, Z7–12 : OAc, Z9–14 : OAc and Z5–10 : OH. Two major types of sensilla were

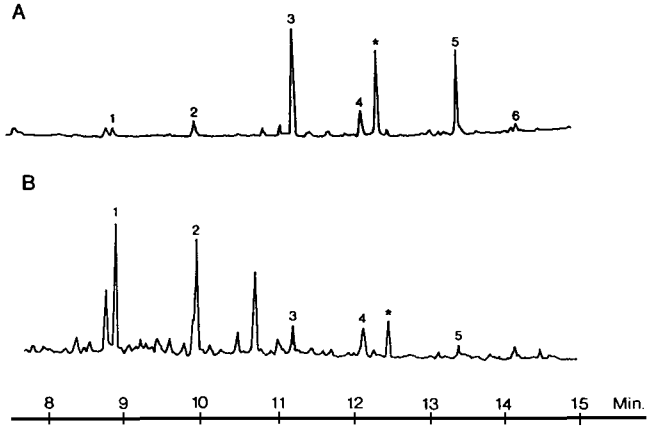

FIG 1. GC-FID chromatogram of female pheromone gland extracts from two populations of *Agrotis segetum*; (A) Swedish, 1 female equivalent; (B) Zimbabwean, 3 female equivalents. Peak 1, Z5–10 : OAc, 2, Z5–10 : OH; 3, Z7–12 : OAc; 4, Z7–12 : OH; 5, Z9–14 : OAc; 6, Z9–14 : OH. *Internal standard Z8–13 : OAc.

found. One type (86% of the sensilla examined) contained a receptor neuron responding to Z5–10 : OAc with a large spike amplitude, together with another receptor neuron responding to Z5–10 : OH with a small spike amplitude. Three of 36 of these sensilla responded also to Z5–12 : OAc with a small spike amplitude. The other sensillum type (14% of sensilla examined) contained one neuron responding to Z7–12 : OAc with a large spike amplitude. In Swedish males, of 30 sensilla examined, the percent of Z7–12 : OAc-type sensilla was 23%. The Z7–12 : OAc-type sensilla in Zimbabwean male antennae were found in the basal and medial part of the antennal branch, while in Swedish male antennae the Z7–12 : OAc-type sensilla could be found along the entire branch (Table 2). Dose–response curves obtained from male Zimbabwean and Swedish *A. segetum* receptor neurons when stimulated with Z5–10 : OAc, Z5–10 : OH, and Z7–12 : OAc (Figure 2A–C) showed a similar response profile between the two populations. However the Zimbabwean receptors showed higher maximal response.

Flight Tunnel Tests. In Zimbabwean *A. segetum*, the flight tunnel assays showed that a blend of Z5–10 : OAc, Z7–12 : OAc, and Z9–14 : OAc at a 1 : 0.25 : 0.03 ratio elicited as many male responses as did the pheromone glands dissected from calling females. The response to this blend was significantly greater than the response to female extracts, which contained high amounts of Z5–10 : OH and Z7–12 : OH (Figure 3). The antagonistic effect of these alcohols was confirmed by a significant decrease in male response to the two-component blend with the addition of the combination of Z5–10 : OH and Z7–12 : OH or the addition of the female extract. Males from the Zimbabwean population were

TABLE 2. SPATIAL DISTRIBUTION OF TWO TYPES OF RECEPTORS ON LATERAL SIDE OF ANTENNAL BRANCHES OF ZIMBABWEAN AND SWEDISH MALE *A. segetum* POPULATIONS RESPONDING TO Z5–10 : OAc OR Z7–12 : OAc

Antennal Region	Sensillum types found (*N*)			
	Zimbabwean population[a]		Swedish population[b]	
	Z5–10 : OAc	Z7–12 : OAc	Z5–10 : OAc	Z7–12 : OAc
Basal	35	11	12	3
Medial	29	4	5	2
Distal	29	0	6	2
Total found	93	15	23	7
% in population	86	14	77	23

[a]Nine branches from seven males tested.
[b]Four branches from four males tested.

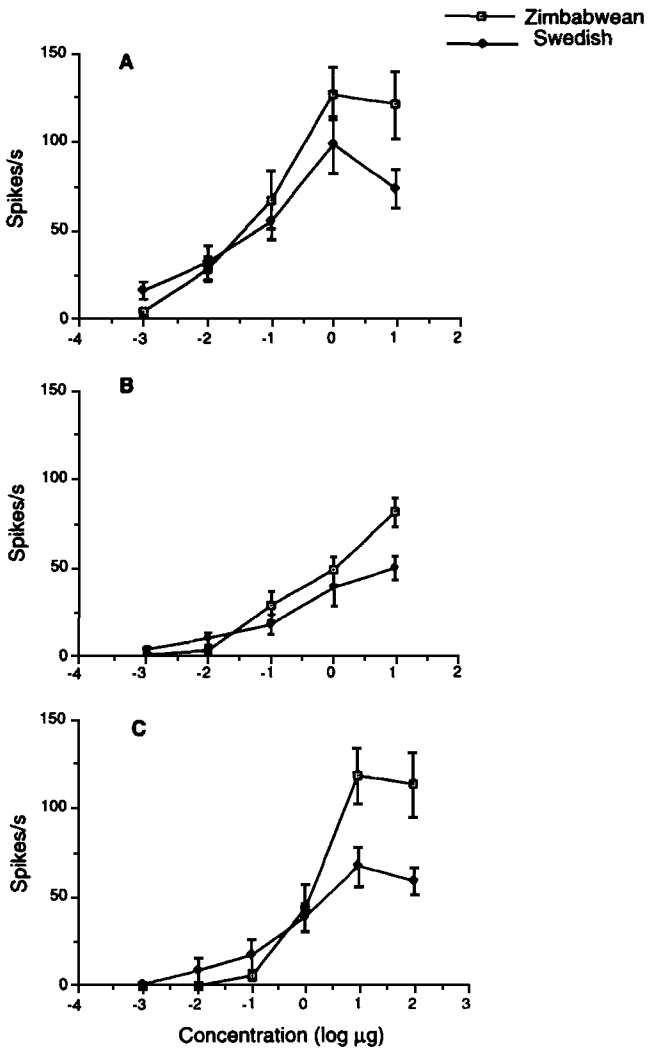

FIG. 2. Dose–response curves of receptor neurons from Swedish and Zimbabwean male *Agrotis segetum* stimulated with Z5–10 : OAc (A), Z5–10 : OH (B), and Z7–12 : OAc (C) (mean ± SEM, N = 10).

significantly more attracted to the Zimbabwean three-component blend (SBC 6) than to the Swedish three-component blend (SBC 1) or to Z5–10 : OAc alone (Figure 4). As in the first flight tunnel experiment (Figure 3), the Zimbabwean two-component blend was also attractive (Figure 4).

Blend	N	Z5-10:OAc (ng)	Z7-12:OAc (ng)	Z9-14:OAc (ng)	Z5-10:OH (ng)	Z7-12:OH (ng)	extracts 3FE	glands 2FE
■	51	-	-	-	-	-	-	+
□	50	-	-	-	-	-	+	-
●	51	0.1	0.025	-	-	-	-	-
△	53	0.1	0.025	0.003	-	-	-	-
○	39	0.1	0.025	-	0.12	0.14	-	-
◆	35	0.1	0.025	-	-	-	+	-

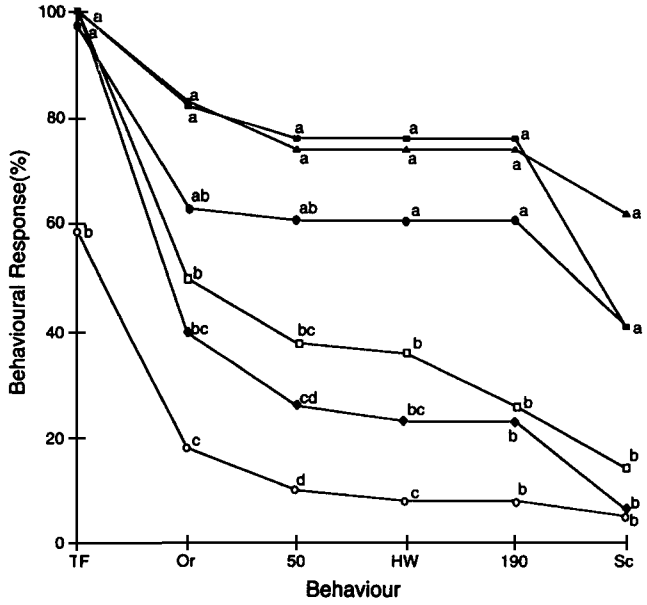

FIG. 3. Upwind behavioral responses of male Zimbabwean *A. segetum* in a flight tunnel to different pheromone stimuli [including Zimbabwean female extracts (3FE) and Zimbabwean female glands (2FE)]. The behaviors are: TF, take off; Or, orientation; 50 cm, moths flying upwind 50 cm from the release point in the plume, HW, moths flying half of the distance from the release point to the source; 190 cm, moths flying from the release point to 10 cm downwind of the source; SC, source contact. Different letters indicate values significantly different at the 95% confidence level, according to the method of adjusted significance levels for proportions (Ryan, 1960). Blends tested are independent of those in Table 1.

Field Tests. Three trapping experiments (numbered 1–3) were carried out in both Sweden and Zimbabwe; two further experiments (4 and 5) were carried out only in Zimbabwe.

Experiments S1 and Z1 tested the relative attractiveness of same-aged vir-

Blend (SBC)	N	Z5-10:OAc	Z7-12:OAc	Z9-14:OAc
		(ng)		
1 ■	30	0.45	2.25	1.13
5 □	51	0.1	0.025	-
6 ◆	53	0.1	0.025	0.003
3 ◇	45	0.1	-	-

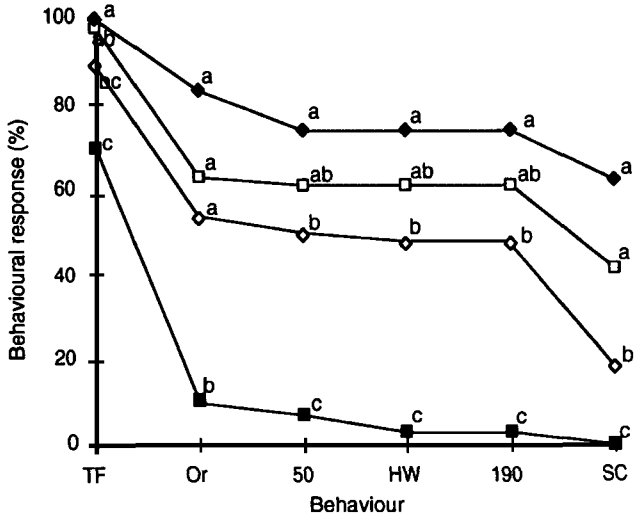

FIG. 4. Upwind behavioral responses of male Zimbabwean *A. segetum* in a flight tunnel to synthetic blends of components. Abbreviations and statistical analyses as in Figure 3, and blend proportions as in Table 1.

gin Swedish and Zimbabwean stock females to local males. In Sweden, Swedish females trapped 95% of the total catch of local males. In Zimbabwe, Swedish females caught only 30% (Table 3, A). The differences in the proportions of males caught by the two types of females at the two localities are statistically significant (Fisher's exact test, two-tailed $P = 1.84 \times 10^{-14}$), indicating a differential response of local males to local vs foreign females. The mean difference ($S - Z$) in catch between members of the pairs of females tested was significant in Sweden (paired t test, two-tailed $P = 0.011$) but not in Zimbabwe ($P = 0.258$) (Table 3, B). The difference in the degree of selectivity exercised by local males for local females was not due solely to the smaller number of pairs tested in Zimbabwe but to a real difference in behavior (supported by an examination of the number of pairs falling into different attractiveness categories (Fisher's exact test, two-tailed $P = 0.003$; Table 3, C).

TABLE 3. RELATIVE ATTRACTIVNESS OF PAIRS OF SAME-AGED VIRGIN FEMALES, ONE SWEDISH (S), ONE ZIMBABWEAN (Z), IN FIELD TESTS IN SWEDEN AND ZIMBABWE

Locality	Total catch (%)				
	Swedish females		Zimbabwean females		
A. Total catch of males, percentages in parentheses					
Sweden	53 (95)		3 (5)		
Zimbabwe	21 (30)		49 (70)		
	Pairs	Mean Diff.	SE	t	P (2-tailed)
B. Analyses of differences (S-Z) in male catches by pairs					
Sweden	8	6.25	1.81	3.45	0.0107
Zimbabwe	6	−4.67	3.66	1.28	0.2580
	Attractiveness category				
	S > Z		S = Z		S < Z
C. Frequencies of pairs in different relative attractiveness categories					
Sweden	8		0		0
Zimbabwe	1		2		3

Experiments S2 and Z2 tested the relative attractiveness of the pairs of bait septa charged with either the Swedish four-component blend (SBC 2) or the Zimbabwean four-component blend (SBC 7). In Sweden, SBC 2 caught 61% of the total local males trapped, while in Zimbabwe, this blend caught 46% (Table 4, A). The differences in the proportions of local males caught by the two blends in Sweden and Zimbabwe are significant (Fisher's exact test, two-tailed $P = 0.003$), indicating a differential response of local males to the synthetic blends matching those of local vs. foreign females. The mean difference ($S - Z$) in catch between members of bait pairs was significantly different in Sweden (paired sample t test, two-tailed $P = 0.0001$) but not in Zimbabwe ($P = 0.3301$) (Table 4, B). In Zimbabwe, local males were apparently less strongly attracted to the local blend than were local males in Sweden (Fisher's exact test, two-tailed $P = 5.06 \times 10^{-4}$; Table 4, C).

In the course of experiment Z2, six of the septa pairs also attracted males of *Chrysodeixis acuta* (Walker) (Noctuidae: Plusiinae). Among all six pairs, SBC 7 caught more males of *C. acuta* than SBC 2. A paired-sample t test of these

TABLE 4. RELATIVE ATTRACTIVNESS OF PAIRS OF BAIT SEPTA CHARGED WITH EITHER SWEDISH FOUR-COMPONENT BLEND (SBC 2) OR ZIMBABWEAN FOUR-COMPONENT BLEND (SBC 7) IN FIELD TESTS IN SWEDEN AND ZIMBABWE

Locality	Total catch (%)	
	SBC 2	SBC 7
A. Total males caught, percentages in parentheses		
Sweden	194 (61)	124 (39)
Zimbabwe	66 (46)	77 (54)

	Pairs	Mean Diff.	SE	t	P (2-tailed)
B. Analyses of differences (S-Z) in male catches by pairs					
Sweden	24	2.92	0.63	4.64	0.0001
Zimbabwe	15	−0.73	0.73	−1.01	0.3301

	Attractiveness category		
	S > Z	S = Z	S < Z
C. Frequencies of pairs in different relative attractiveness categories			
Sweden	20	2	2
Zimbabwe	4	2	9

data indicated that SBC 7 was significantly more attractive for *C. acuta* than was SBC 2 ($0.02 > P > 0.01$).

Experiments S3, Z3a, and Z3b tested the relative attractiveness of six pheromone component blends: SBC 1, 2, 3, 5, 6, and 8 (Table 1). In Zimbabwe the same experiment was carried out on two different occasions (designated experiments Z3a and Z3b). A chi-square test of homogeneity of the frequencies obtained in the two Zimbabwean experiments suggests that these two data sets do not represent random samples from the same population ($P = 0.005$). In Sweden, the most attractive blend was the Swedish four-component blend (SBC 2); however, responses to SBC 1, 6, and 8 were not significantly different from the response to SBC 2 (Figure 5). SBC 3 (Z5–10 : OAc alone) was the least attractive one in Sweden. In Zimbabwe, the Zimbabwean three-component blend (SBC 6) consistently caught most male moths, while the Swedish three-component blend (SBC 1) and Zimbabwean two-component blend (SBC 5) were the least attractive (Figure 5). The results showed the high relative attractiveness of Z5–10 : OAc alone (SBC 3) for Zimbabwean males as well as the very strong

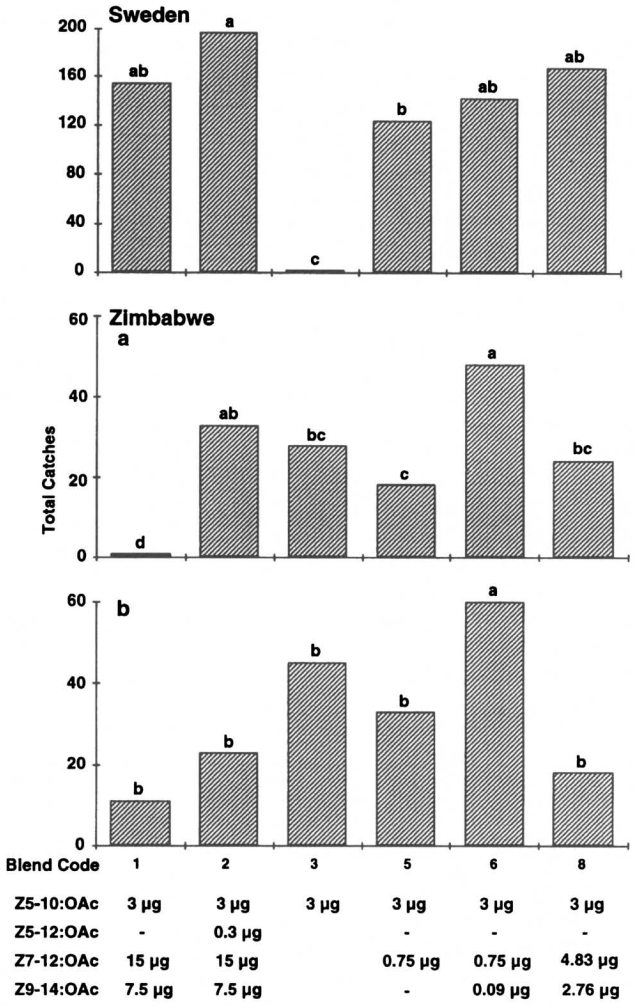

FIG. 5. Field trapping of male *A. segetum* in Sweden and in two separate trials in Zimbabwe (a and b) to six synthetic pheromone blends (experiments S3 and Z3) ($N = 5$). The same letters within each series indicate values that are not significantly different according to ANOVA [$\ln(x + 1)$ transformation] followed by the FPLSD test ($P < 0.05$).

discrimination by Zimbabwean males between the Swedish three- (SBC 1) and four-component (SBC 2) blends.

In two experiments conducted only in Zimbabwe, five blends (SBC 2, 3, 4, 6, and 7 (Table 1) were tested (experiment Z4), while three blends (SBC 1, 6, and 7) were each tested at four dosages (experiment Z5). Although the

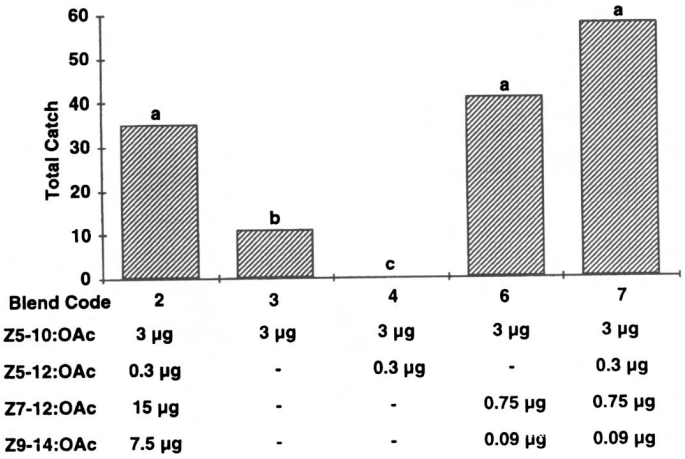

FIG. 6. Field trapping of male *A. segetum* in Zimbabwe to test the effect of *Z*5–12 : OAc (experiment Z4) ($N = 5$). The same letters within each series indicate values that are not significantly different according to ANOVA [$\ln(x + 1)$ transformation] followed by the FPLSD test ($P < 0.05$).

response was highest to the Zimbabwean four-component blend (SBC 7) in Z4 and at three of four doses in Z5, the responses to SBC 7 were never significantly different from the responses to the Zimbabwean three-component blend (SBC 6) (Figures 6 and 7). In experiment Z4, *Z*5–10 : OAc alone (SBC 3) and *Z*5–10 : OAc plus *Z*5–12 : OAc (SBC 4) were clearly the least attractive (Figure 6). In experiment Z5, the optimum catches for all three blends were at a dose of 3 µg of *Z*5–10 : OAc in field trapping in Zimbabwe (Figure 7).

DISCUSSION

The present study confirms that a Zimbabwean population of *A. segetum* has pheromone-emitting and -receiving characteristics that are distinctly different from those of European populations (Tóth et al., 1992). The pheromone characteristics of the Zimbabwean population are, however, rather different from those reported in the original survey. This discrepancy may involve the proportions of components used by Tóth et al. (1992) for their ternary mixture. Gas chromatographic analyses of Zimbabwean female gland extracts showed that the three major components (*Z*5–10 : OAc, *Z*7–12 : OAc, and *Z*9–14 : OAc) are in the proportions 1 : 0.25 : 0.03 as against the proportions 1 : 4.8 : 2.5 found in gland extracts of Swedish females. The Swedish proportions are in agreement with those reported previously (Löfstedt and Odham, 1984; Löfstedt et al., 1985b).

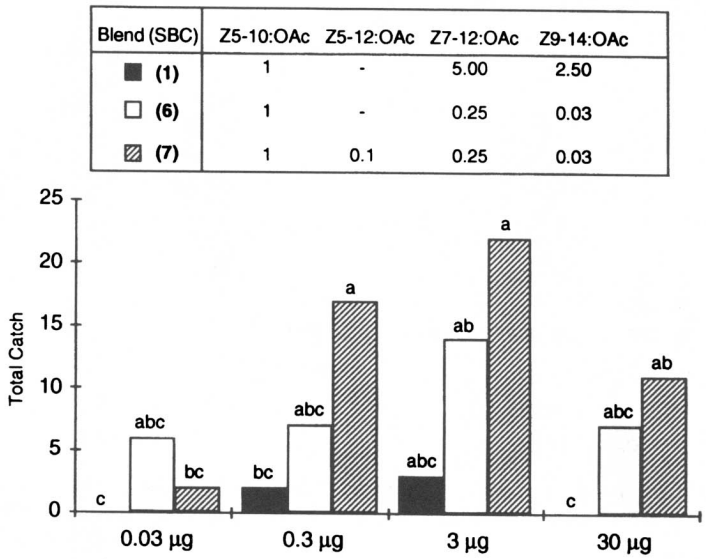

FIG. 7. Field trapping of male *A. segetum* in Zimbabwe with four different doses of three blends (Experiment Z5) ($N = 5$). Dosage refers to the amount of Z5–10 : OAc. Same letters within each series indicate values that are not significantly different based on ANOVA [$\ln(x + 1)$ transformation] followed by the FPLSD test ($P < 0.05$).

In addition, female gland extracts from both populations show traces of a fourth component with a retention time corresponding to Z5–12 : OAc. This component has been reported previously (Löfstedt et al., 1986) and has been shown to be both electrophysiologically and behaviorally active in Swedish *A. segetum* (Wu et al., 1995). In flight tunnel assays, Zimbabwean males were significantly more attracted to the Zimbabwean three-component blend than to the Swedish three-component blend or Z5–10 : OAc alone. Field pairwise comparison tests suggested that Swedish males have a stronger selectivity for local females and blends than do Zimbabwean males for local females and blends. Zimbabwean males do, however, select markedly against the Swedish three-component blend or the hybrid blend in both flight tunnel and field tests. The difference between these two blends and Zimbabwean blends is the proportions of the second and third components (Z7–12 : OAc and Z9–14 : OAc, respectively). The higher proportions of one or both of these compounds in the Swedish blend seems to be responsible for their decreased attractiveness to Zimbabwean males. Nevertheless, the presence of quite small amounts of either one or both of the second and third components in the Zimbabwean three-component blend significantly enhances its attractiveness over Z5–10 : OAc alone. When Tóth et al. (1992) first reported the existence of populations of *A. segetum* with different charac-

teristics in southern and eastern Africa, they characterized them as being most strongly attracted to Z5–10 : OAc alone. However, the component ratio (1 : 1 : 8) used for comparison deviated widely from what is now known of the pheromone composition of the Zimbabwean population. In light of the present experiments, the high amounts of one or both of Z7–12 : OAc and Z9–14 : OAc would be expected to have an antagonistic effect on Zimbabwean male attractivity. The effect of the fourth component (Z5–12 : OAc) is particularly interesting. When added to Z5–10 : OAc, it significantly decreased attractivity for Zimbabwean males. In contrast, when added to the Zimbabwean three-component blend to produce the four-component blend, it did not statistically increase attractiveness even though an examination of the catches does suggest some enhancement. In further striking contrast, when added to the Swedish three-component blend to produce the Swedish four-component one, it caused a statistically significant increase in attractiveness for Zimbabwean males, bringing the four-component Swedish blend virtually into the ranges of attractiveness (for Zimbabwean males) of the Zimbabwean three-component blend and even the Zimbabwean four-component blend. Apparently, the presence of small amounts of the fourth component (Z5–12 : OAc) in some way overcomes the loss of attractivity to Zimbabwean males caused by the presence of the higher concentrations of one or both of the second (Z7–12 : OAc) and third (Z9–14 : OAc) component in the Swedish blends. In the total absence of the second and third components, the fourth component in high concentrations reduced attractivity for Zimbabwean males.

Although it was not possible to demonstrate, by single sensillum recordings, a definite difference in the proportions of Z5–10 : OAc-type receptor neurons to Z7–12 : OAc-type receptors on Swedish as against Zimbabwean male antennal branches, there was a suggestion that in Zimbabwean males the latter type of neurons was confined to the basal and medial thirds of the antennal branches, whereas it was present along the whole branch length in Swedish males. The overall proportions of Z7–12 : OAc-type sensilla are similar to those previously reported (Löfstedt et al., 1982, 1986). It has earlier been reported that different physiologically defined sensillum types are present at certain locations on the male antennae. In *Agrotis exclamationis*, receptor cells in the medially situated sensilla responded to totally different pheromone components from receptor neurons in the lateral sensilla (Hansson et al., 1986). In *Spodoptera littoralis* sensilla specifically tuned to Z9,E11–14 : OAc were evenly distributed over the ventral antennal surface, while the second sensillum type containing one neuron responding to Z9,E12–14 : OAc and another neuron responding to Z9–14 : OH was only found along the lateral part of the antennal segments (Ljungberg et al., 1993). Likewise, in *Trichoplusia ni* sensilla tuned to Z9–14 : OAc and 12 : OAc were reported to be located in the middle subsegments of the antenna away from the lateral margins and within a row of hairs (Todd et al., 1993). In the present study, we found that the Z7–12 : OAc sensillum type was situated in the basal and

medial antennal branches on the male antennae of Zimbabwean turnip moths. If the number of sensilla and the distribution of particular sensillum types are proved to be consistent among individuals from one population, it will be possible to construct a map illustrating precisely the location of different sensilla on the male antennae of each populations. This accomplishment will be of great use for further studies of a particular sensillum type.

The antagonistic effect of Z5–10 : OH has previously been demonstrated in the Swedish population (Löfstedt et al., 1985a). The present experiments demonstrate a significant reduction in the behavioral responses observed in flight tunnel assays when this compound was present in female gland extracts or in active synthetic blends and also that it is a behavioral antagonist for the Zimbabwean population. Although Z5–10 : OH was found in the female gland extracts in both populations, it is not known whether it is emitted by the female glands during calling. It has been shown in the present study that the glands themselves are not antagonistic to males. Analyses of airborne volatile collections from glands therefore need to be done in the future.

Taking into account the relatively large amounts of the second and third components in the Swedish blends that can apparently be "neutralized" for the Zimbabwean male by a relatively small amount of the fourth component, it seems unlikely that the sensory integration occurs at the level of the primary receptor neurons. Integration is much more likely to reside in the central nervous system at levels where the sensory stimuli from the different neurons are coordinated. Antennal lobe neurons that respond only to the blend, and not to any single component, as well as neurons that recognize the differences in ratios between pheromone components were found in *A. segetum* (Wu et al., 1996). These results indicate that pheromone discrimination between the two populations can be partially achieved at the antennal lobe level.

Differences in the ratios of homologous acetates in the pheromones of different geographical populations have also been reported for *Zeiraphera diniana* (Guerin et al., 1984) and *Planotortrix exessana* (Foster et al., 1986, 1989), but their evolutionary interpretation is not yet understood. From our present study, there is no evidence for the idea that interpopulational differences have been brought about by reproductive character displacement resulting from the presence of species using similar pheromone signals. It is interesting, in this context, that the sympatric and sporadically numerous pest species *Chrysodeixis acuta* was more attracted to the Zimbabwean four-component blend of the sympatric Zimbabwean population than it was to the Swedish four-component blend.

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DISCRIMINATION BETWEEN SELF-PRODUCED PHEROMONES AND THOSE PRODUCED BY INDIVIDUALS OF THE SAME SEX IN THE LIZARD *Cordylus cordylus*

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Abstract—Male and female *Cordylus cordylus* can discriminate between tiles labeled by their own pheromones and tiles labeled by individuals of the same sex, as shown by elevated tongue-flick rates in which the tongue contacts only air above the tiles labeled by other individuals and tongue-flicks in which the tongue contacts the tiles themselves. Potential pheromone sources for these discriminations are the femoral glands, cloacal glands, generation glands, ventral skin, and excretory products. Although studied in few species, pheromonal discriminations between self and other individuals, familiar and unfamiliar individuals, and kin and unrelated individuals appear to be broadly distributed in lizards, occurring in Iguania and in both Gekkonoidea and Scincomorpha within Scleroglossa. Both sexes of *C. cordylus* defend territories against both sexes. An ability to distinguish pheromones of other individuals of the same sex from self-produced pheromones would allow detection of intruders, but pheromonal discriminations among individuals would be more useful. Adaptive functions of pheromonal discriminations for residents and nonresidents and discriminatory abilities required are discussed. Tongue-flicks touching labeled tiles differed between experimental conditions, suggesting vomerolfactory discrimination, but the interpretation of a similar difference for tongue-flicks that contacted no substrate is problematical. Such air tongue-flicks might indicate sampling of volatile molecules for delivery to the vomeronasal organs. Experiments are needed to conclusively determine the sensory bases of the discriminations and the role of air tongue-flicks.

Key Words—Behavior, pheromone, self-recognition, tongue-flicking, Squamata, Cordylidae.

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INTRODUCTION

Squamate reptiles use their chemical senses to investigate diverse stimuli of biological importance, especially those bearing on feeding, predation avoidance, and social behavior. In lizards, pheromonal communication is important for location and recognition of conspecifics, sex, and reproductive condition (Cooper and Vitt, 1986a–c; 1987a,b; Cooper et al., 1986; Cooper and Garstka, 1987). Studies of lizard pheromonal communication have established that species representing diverse families are capable of detecting and responding selectively to pheromones produced by conspecifics (reviewed by Mason, 1992; Cooper, 1994). In all families studied to date, some discriminations, especially the ability to discriminate pheromonally between conspecific males and females, have been detected (references in Cooper, 1994; Cooper et al., 1994, 1996; Cooper and Steele, 1997).

Less well known are the abilities of lizards to make finer discriminations, such as those between self-produced pheromones and those produced by other individuals of the same sex, those between two other individuals of the same or opposite sex, and those between kin and unrelated individuals. There is evidence for all of these abilities in one or more species (e.g., Graves and Halpern, 1991; Alberts, 1992; Alberts and Werner, 1993; Bull et al., 1993, 1994; Cooper, 1996; Main and Bull, 1996; López et al., 1997; Steele and Cooper, 1997), but the taxonomic distribution of the abilities and their relationships to social behavior remain obscure.

An ability to distinguish their own pheromones from those of other individuals of the same sex upon encountering pheromone deposits of unknown origin is a prerequisite for pheromonal recognition of rivals. The potential importance of such ability has been suggested for male lizards (Glinski and Krekorian, 1985; Olsson, 1994) and would seem to apply to females as well. Assessment of the presence of other individuals of the same sex may be important for lizards that pheromonally mark and attempt to exclude other consexual individuals from territories, monitor the activities of neighbors, or detect the presence of sexual rivals during breeding periods (e.g., Simon, 1983; Duvall et al., 1987; Graves and Halpern, 1991). It may be equally important to transients for determining the residence status or dominance of other lizards.

Cordylidae is a small family of lizards endemic to southern Africa for which little is known about pheromonal communication. However, the only previous study established that one species, *Cordylus cordylus*, is capable of detecting conspecific pheromones and responding differentially to male and female pheromones (Cooper et al., 1996). Male *C. cordylus* detected pheromones deposited by both sexes of conspecifics on ceramic tiles and exhibited higher tongue-flick rates in response to female than to male pheromones (Cooper et al., 1996), demonstrating an ability to discriminate between the pheromones of

the sexes. Females also tongue-flicked at higher rates in response to conspecific pheromones of both sexes than to an odorless control, but the elevation was significant only in response to female pheromones (Cooper et al., 1996). However, females tongue-flicked female pheromones more frequently than male pheromones, demonstrating pheromonal detection and probably identification of conspecific females (Cooper et al., 1996).

In this paper we present an experimental study of the ability of both sexes of a cordylid lizard, *C. cordylus*, to respond differentially to its own pheromones and those produced by another individual of the same sex presented on labeled substrates. Although diverse behaviors may indicate detection of pheromones (e.g., Greenberg, 1943; Cooper et al., 1986; Cooper and Steele, 1997; Bull et al., 1993; Steele and Cooper, 1997), tongue-flicking is the most widely used behavioral index of responses to pheromones in lizard studies because it provides a convenient objective index of chemosensory investigation (Burghardt, 1970). We discuss deposition sites and sources of pheromones, the adaptive significance and possible mechanisms of discriminations based on scent marks, and the sensory basis for the discriminations.

METHODS AND MATERIALS

Subjects and Maintenance. Adult *C. cordylus* were collected in the Western Cape Province of South Africa in March 1993 and transported to the University of Stellenbosch. They were housed individually in glass terraria of varying size, each of which contained a water bowl and a food bowl on a gravel substrate. Water was continuously available; the staple diet was mealworms, which were supplemented by termites and other insects periodically. Each cage included a horizontal crevice made of two ceramic tiles (24 × 12 × 1 cm) separated by wood strips (12 × 2 × 1.7 cm). The natural regional light cycle was maintained by light through a window and temperature was fixed during the experiments at 30°C.

Experimental Procedures and Design. Previous work established that *C. cordylus* responds to conspecific pheromones deposited on ceramic tiles by increased tongue-flick rates (Cooper et al., 1996). In the present study we observed tongue-flicking responses to ceramic tiles labeled by either the same individual being tested or by another individual of the same sex. Tiles washed in Alconox detergent were placed in home cages as crevices. Animals were allowed to deposit pheromones on the upper surfaces of the lower ceramic tiles in the crevices in their home cages for at least one week prior to use of the labeled tiles as stimulus sources. The lizards spent much time in the crevices even during the daylight hours, with their ventral surfaces in contact with the tiles. This provided ample opportunity for transfer of pheromones from pos-

sible ventrally located sources such as femoral glands, generation glands, cloaca, and skin.

Using randomized blocks designs, we tested the responses of each of 10 males and 15 females to self-labeled ceramic tiles or to tiles labeled by another individual of the same sex. Each lizard was tested under both conditions in counterbalanced sequence. One individual of each sex was discarded due to immobility throughout the trials, giving final sample sizes of nine for males and 14 for females. For males the trial sequence was counterbalanced for the first eight individuals and selected randomly for the ninth. For females the sequence was completely counterbalanced. Some tiles bearing pheromonal stimuli from other individuals were taken from home cages of individuals not tested.

Data were collected on April 12–15, 1993, from 07:57 to 17:44 hr for males and from 09:52 to 14:28 hr for females. All lizards were active at the times tested. Prior to each trial, a stimulus tile was placed in a test chamber (62- × 32- × 33-cm glass terrarium) having a gravel substrate and positioned at the midpoint of the end nearer the observer during data collection with one edge in contact with the arena's wall. The arena was located 0.1 m from a panel of one-way glass that permitted observation from a darkened adjacent room.

Prior to each trial, the experimenter transported the lizard from its home cage to the test arena and positioned it in the middle of the cage ca. 15 cm from the end of the arena in contact with the tile. Pilot tests showed that the lizards often flee to the far end of the cage if released immediately. To reduce the frequency of such escape attempts that required the trial to be conducted at a later time, the experimenter completely covered the lizard with his hand, pressed it gently against the substrate, and removed his hand only when the lizard stopped trying to escape. Upon releasing the lizard, the experimenter withdrew to the adjacent observation room. After a variable period of immobility, the lizard would start to explore the arena, typically walking slowly and tongue-flicking frequently. Data recorded were the number of tongue-flicks in 10 min starting when the first tongue-flick touched the tile. Two separate categories of tongue-flicks were recorded. Substrate tongue-flicks contacted the tiles, while air tongue-flicks were performed while a lizard's head was over the tile, but the tongue did not contact any surface. If a lizard did not move within 30 min, it was returned to its home and dropped from the experiment.

Statistical Analysis. Data were analyzed by single-factor analysis of variance for a randomized blocks design (Sokol and Rohlf, 1995). Separate tests were conducted for substrate tongue-flicks, air tongue-flicks, and pooled substrate and air tongue-flicks. Prior to analysis, the assumption of homogeneity of variance was examined by using Hartley's F_{max} test (Winer, 1962). Where significant heterogeneity of variance was detected, the data were logarithmically transformed to obtain homogeneity before conducting ANOVA. Significance tests were two-tailed with $\alpha = 0.05$.

RESULTS

General Responses to Experimental Conditions. Both sexes tongue-flicked frequently while exploring the test arena and while on the labeled tiles. Upon initially contacting tiles labeled by other individuals, some lizards exhibited obvious increases in tongue-flicking rates, but this was not quantified due to lack of data during exploration off the tiles. No overt responses to tiles other than tongue-flicking were observed. In particular, no males or females made any obvious efforts to rub their ventral surfaces on labeled tiles. One female urinated on a self-labeled tile.

Male Data. All nine males performed both air and substrate tongue-flicks in each condition, with the sole exception of one male that did not perform an air tongue-flick over a self-labeled tile. Males tongue-flicked substantially more frequently in response to tiles labeled by other individuals than to self-labeled tiles (Table 1). They performed somewhat fewer air tongue-flicks than substrate tongue-flicks, especially in response to tiles labeled by other males (Table 1).

Variances for total tongue-flicks were significantly heterogeneous for the raw data ($F_{max} = 8.91$; $df = 2, 8$; $P < 0.01$), but homogeneous following transformation ($F_{max} = 1.12$; $df = 2, 8$; $P > 0.05$). Tiles labeled by other males elicited significantly more total tongue-flicks than did self-labeled tiles ($F = 13.38$; $df = 1, 8$; $P < 0.01$.

Substrate tongue-flick data exhibited pronounced heterogeneity of variance

TABLE 1. NUMBERS OF TONGUE-FLICKS BY ADULT MALE *Cordylus cordylus* RESPONDING TO TILES LABELED BY THEMSELVES AND CONSPECIFICS OF THE SAME SEX

	Source of label	
	Self	Conspecific
Substrate tongue-flicks		
Mean	4.3	14.4
SE	1.0	4.2
Range	1–11	4–44
Air tongue-flicks		
Mean	3.1	8.0
SE	0.9	1.6
Range	0–8	3–15
Total tongue-flicks		
Mean	7.4	22.4
SE	1.8	5.4
Range	1–19	7–58

(F_{max} = 17.46; df = 2, 8; $P < 0.01$), which was removed by logarithmic transformation (F_{max} = 1.62; df = 2, 8; $P > 0.05$). Males performed significantly more substrate tongue-flicks in response to tiles labeled by other males than to self-labeled tiles (F = 9.88; df = 1, 8; $P < 0.02$).

Variances of data from air tongue-flicks, although somewhat greater when responses were to stimuli from other males (nearly double), were not significantly heterogeneous (F_{max} = 3.33; df = 2, 8; $P > 0.05$). Males performed significantly more air tongue-flicks in response to tiles labeled by other males than to self-labeled tiles (F = 14.47; df = 1, 8; $P < 0.006$).

Female Data. The 14 females all performed both types of tongue-flicks in each condition with the sole exception of a female that did not perform an air tongue-flick over a self-labeled tile. Like males, the females tongue-flicked much more frequently in response to tiles labeled by other individuals than to self-labeled tiles and performed fewer air tongue-flicks than substrate tongue-flicks (Table 2). Female and male tongue-flick rates were very similar.

Variances of data from total, substrate, and air tongue-flicks were homogeneous (F_{max} = 1.64, 1.43, and 2.08, respectively; df = 2, 13; $P > 0.05$ each). Females performed significantly more total tongue-flicks when responding to tiles labeled by other females than to their own tiles (F = 16.03; d = 1, 13; $P < 0.005$. Tiles labeled by other females elicited significantly more substrate tongue-flicks (F = 13.66; df = 1, 13; $P < 0.005$) and air tongue-flicks (F = 10.13; df = 1, 13; $P < 0.01$) than did self-labeled tiles.

TABLE 2. NUMBERS OF TONGUE-FLICKS BY ADULT FEMALE *Cordylus cordylus* RESPONDING TO TILES LABELED BY THEMSELVES AND CONSPECIFICS OF THE SAME SEX

	Source of label	
	Self	Conspecific
Substrate tongue-flicks		
Mean	7.0	15.8
SE	1.9	2.2
Range	1–28	2–29
Air tongue-flicks		
Mean	4.4	9.0
SE	0.8	1.1
Range	1–11	2–15
Total tongue-flicks		
Mean	11.4	25.0
SE	2.4	3.1
Range	2–34	10–42

DISCUSSION

Pheromonal Discriminations, Deposition Sites, and Sources. The significantly greater tongue-flick rates when responding to tiles labeled by other individuals indicate that both sexes discriminated between pheromones produced by themselves and other individuals of the same sex. The same conclusion is well supported for both sexes by the separate analyses of air and substrate tongue-flicks and by that for the pooled tongue-flicks.

Like many other cordylids, *C. cordylus* makes extensive use of rock crevices as refuges from predators and thermal extremes, and as sleeping sites, and very often perch on adjacent rocks while basking and foraging (Burrage, 1974; Branch, 1988; personal observations). Thus, tiles from home crevices may be a realistic experimental model for elicitation of chemosensory investigation of conspecific pheromones.

Sites of pheromone synthesis and secretion in cordylids have not been investigated, but several potential pheromone sources are located in ventral positions well suited to deposition on rocks. The femoral glands of cordylids secrete waxy plugs that are enlarged during the breeding season in males (Van Wyk, 1990; Van Wyk and Mouton, 1992). Their protrusion below the femoral region ensures contact with and deposition on substrates. Femoral gland secretions have known importance for pheromonal communication in two species of iguanid lizards. Male desert iguanas, *Dipsosaurus dorsalis*, can discriminate between their own femoral gland secretions and those of other males (Alberts, 1992). Male green iguanas, *Iguana iguana*, can discriminate between familiar and unfamiliar males based solely on their femoral gland secretions (Alberts and Werner, 1993).

Cloacal glands are another possible source of pheromones in cordylids. Scincid lizards lack femoral glands producing waxy secretion plugs, but have pheromonally active cloacal glands (Cooper et al., 1986; Trauth et al., 1987). Two scincid species are known to make pheromonal discriminations between themselves and other individuals (*Tiliqua scincoides*, Graves and Halpern, 1991) and familiar and unfamiliar individuals (*Eumeces laticeps*; Cooper, 1996). Cordylids, which form a portion of the sister taxon of Scincidae, possess similar cloacal glands (Mackay, 1993). Urine or feces might carry intrinsic pheromones, or pheromones produced in specialized cloacal glands.

Other possible sources of pheromones important for self-other discriminations that would lend themselves to deposition by ventral contact with rocks or tiles are the ventral skin and generation glands. Generation glands are modified secretory patches of scales found in several interspecifically varying locations on the surfaces of cordylids (Van Wyk and Mouton, 1992). Generation glands in *C. cordylus* occur on the ventral thigh anterior to the femoral glands (Van Wyk and Mouton, 1992). They are present in males, but in female *C. cordylus* are found only in some lowland populations, being absent in montane areas

(Van Wyk and Mouton, 1992). Differentiation of generation glands coincides with sexual maturation in another cordylid, *Pseudocordylus melanotus* (Mouton and Van Wyk, 1993), hinting at a possible role in pheromonal communication. Generation glands also are active in neonatal *C. macropholis* (Mouton, unpublished data) when steroid levels are higher than in older juveniles. Pheromonal properties of morphologically unspecialized ventral skin and of generation glands have not been investigated. Due to presence of numerous potential sites of pheromone secretion, cordylids are an ideal group for investigation of pheromone sources and information content.

The relatively few studies of pheromonal discriminations between self and other individuals, between familiar and unfamiliar individuals, and between kin and unrelated individuals show that species in several of the major lizard clades are capable of some degree of individual recognition mediated by chemical senses. One or more of these abilities have been demonstrated in both Iguania (e.g., Alberts, 1992; Alberts and Werner, 1993) and Scleroglossa. Within Scleroglossa, these abilities have been demonstrated experimentally in a gekkonoid lizard species (Steele and Cooper, 1997) and several scincomorphan species, including representatives of Scincidae (e.g., Bull et al., 1994; Cooper, 1996; Main and Bull, 1996) and Cordylidae (this paper). Such discriminations may occur in a wide diversity of lizards.

Adaptive Significance and Mechanism of Pheromonal Self-Recognition. The social system of *C. cordylus* is poorly known, but Burrage (1974) reported that individuals defend their retreats in crevices or under rocks and that territoriality by both sexes, including defense of a larger portion of the home range, occurs at the periphery of rock koppies and in sandy areas with isolated rocks. Active or passive scent-marking of rocks presumably occurs throughout the territory and especially in the heavily used vicinity of the retreat.

Residents must frequently encounter their own scent-marks throughout their territories. An ability by the resident to distinguish its own scent-marks from those of other individuals of the same or other sex could provide socially valuable information about the presence of transient individuals. In this study we have demonstrated an ability only to discriminate between self and others of the same sex, which by itself would be insufficient to provide information about differences between neighbors and transients of the same sex or between the opposite-sex resident and other individuals of the opposite sex.

Given the defense by residents of both sexes against nonresidents of both sexes, more sophisticated abilities, such as individual recognition or ability to discriminate between familiar and unfamiliar individuals of both sexes would be more efficient. Some lizards, including *E. laticeps* (Cooper, 1996) and *I. iguana* (Alberts and Werner, 1993) can make some of these more precise distinctions based on chemical cues sampled from substrates. Further study is needed to investigate the finer abilities in cordylids.

Nonresidents, which are subject to vigorous attack by residents of either sex (Burrage, 1974), might gain information about the identity and perhaps other characteristics of the residents from pheromones deposited by them. Thus, residents might mark their territories to provide information enabling nonresidents to avoid conflicts. Matching scent-marks deposited in the vicinity with those of an encountered individual may be an important mechanism by which nonresident mammals avoid conflict with the resident (Gosling, 1982; Gosling and McKay, 1990a,b). Communication of such information by lizards would be much more efficient if they can recognize differences among individuals pheromonally, not merely differences between the two categories of self and other individuals.

Sensory Roles. Tongue-flicking is an important means of gathering chemical samples to be evaluated by vomerolfaction (Gillingham and Clark, 1981; Graves and Halpern, 1989; Cooper and Burghardt, 1990). Vomerolfactory analysis is necessary for normal responses to sex pheromones in snakes. Male vipers (*Vipera berus*) with anesthetized vomeronasal organs (Andrén, 1982) and male garter snakes (*Thamnophis radix*) with severed vomeronasal nerves (Kubie et al., 1978) do not court sexually attractive females. Vomerolfaction may be important in lizards.

The skink *Tiliqua rugosa* (Bull et al., 1993), appears to be able to locate conspecifics using airborne cues. The primary sense used for such location is very likely olfaction rather than vomerolfaction because olfaction is sensitive to volatile substances, whereas vomerolfaction is sensitive primarily to nonvolatile substances sampled by contact with substrates (reviewed by Halpern, 1992). The same species can locate conspecifics by scent-trailing mediated by tongue-flicking (Bull et al., 1993). Both *T. rugosa* and another Australian skink, *Egernia stokesii*, tongue-flick at higher rates to chemical cues from their own offspring than to cues from offspring of other females (Bull et al., 1994; Main and Bull, 1996). The latter discriminations are presumably vomerolfactory.

The elevated rates of tongue-flicks contacting tiles by *C. cordylus* in response to pheromones produced by other individuals suggest vomerolfactory discrimination, but the interpretation of tongue-flicks that did not contact any substrate is less clear. According to the Cowles and Phelan (1958) hypothesis, volatile substances detected by olfaction induce increased tongue-flicking rates for finer discriminations made by vomerolfaction. If contact with a substrate is needed to sample chemicals for transfer to the vomeronasal organs, what can be the function of air tongue-flicks? Many lizards perform air tongue-flicks that involve extensive lingual protrusion and sweeping of the tongue upward and outward, then downward prior to retraction (Gove, 1979; personal observations). It is thus unlikely that air tongue-flicks are simply inaccurate ones that miss targeted substrates.

The vomeronasal organs of turtles can detect both volatile and nonvolatile molecules (Shoji and Kurihara, 1991), suggesting the possibility that vomerol-

faction may contribute to chemosensory assessment of some volatile substances in squamate reptiles. If so, air tongue-flicks by *C. cordylus* might indicate that volatile substances present in the pheromone deposits elicit air tongue-flicking. Another possibility, a sort of reverse Cowles-Phelan hypothesis, is that detection of nonvolatile pheromonal elements by vomerolfaction induces air tongue-flicking and perhaps olfactory sampling for analysis of possible associated volatile components. Experimental studies are needed to elucidate the roles of air tongue-flicks.

Whether or not pheromonal discriminations may be based on other chemical senses in a given squamate taxon, differential tongue-flick rates provide presumptive evidence for vomerolfactory discriminations. However, roles of vomerolfaction and other chemical senses must be demonstrated experimentally by studying effects of selectively blocking senses on chemically mediated discriminations (Halpern, 1992).

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ALLELOPATHIC POTENTIAL OF AQUATIC PLANTS ASSOCIATED WITH WILD RICE (*ZIZANIA PALUSTRIS*): I. BIOASSAY WITH PLANT AND LAKE SEDIMENT SAMPLES

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Abstract—The allelopathic potential of eight aquatic plants associated with wild rice was investigated using lettuce and wild rice seedling bioassays. Rhizome aqueous extracts of *Scirpus acutus*, *Potamogeton natans*, *Nymphaea odorata*, *Nuphar variegatum*; shoot extract of *Eleocharis smallii*; whole plant extract of *Myriophyllum verticillatum*; and leaf extract of *P. natans* significantly reduced the root length of lettuce and wild rice seedlings. The lettuce seedling bioassay was more sensitive than the wild rice bioassay. Shoot growth was less affected than the root growth. Water extract of sediments associated with the aquatic plants had little growth inhibitory effect on wild rice. Our study did not yield any conclusive evidence that the wild rice-associated aquatic plants have allelopathic effects on wild rice. We emphasize the use of target species as a bioassay material in allelopathic studies. Further investigation on allelopathic effects of lake sediments associated with the neighboring plants of wild rice is necessary to evaluate their ecological significance.

Key Words—Allelopathy, aquatic plants, growth inhibition, lake sediments, seedling bioassay, wild rice, *Zizania palustris*, *Eleocharis smallii*, *Equisitum fluviatile*, *Myriophyllum verticillatum*, *Nuphar variegatum*, *Nymphaea odorata*, *Potamogeton natan*, *Scirpus acutus*, and *Sparganium fluuctuans*.

INTRODUCTION

Wild rice, *Zizania palustris* L. an annual aquatic cereal crop grows in natural lakes throughout eastern and north-central North America. A number of other

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aquatic plants have been reported to grow within the natural stands of wild rice and are thought to affect its growth (Aiken et al., 1988). Lee (1982) reported that emergent species such as spike-rush (*Eleocharis smallii* L.), big bulrush (*Scirpus acutus* Muhlenb), and horsetail (*Equisetum fluviatile* L.) displace wild rice from shallow waterbodies. Free-floating and submerged species such as white water lily (*Nymphaea odorata* Aiton), yellow water lily (*Nuphar variegatum* Engelm), bur reed [*Sparganium fluctuans* (Morong) Robinson], *Ceratophyllum* spp., and *Myriophyllum* spp. are also reported to be detrimental to wild rice (Aiken et al., 1988).

The adverse effects of aquatic plants on wild rice may result from competition for light, nutrients, and space or from allelopathic interference (Lee, 1987). Since spike-rush and big bulrush have a much lower leaf area and biomass than wild rice, competition for light seems unlikely. In a previous study Quayyum et al. (unpublished data) could not find any significant difference in inorganic nutrients between lake sediments with and without wild rice associated plants. It is therefore, hypothesized that allelopathy may be involved in this growth inhibition process.

Allelopathic interactions in terrestrial plants are reported by many authors (Rice, 1984; Putnam and Tang, 1986; Rizvi and Rizvi, 1992; Mallik, 1987, 1992; Mallik and Roberts, 1994), but relatively few studies have been reported involving aquatic plants (Gopal and Goel, 1993).

Different species of spike-rush have been reported as allelopathic to other aquatic plants (Nichols and Shaw, 1983; Yeo and Thurston, 1984; Ashton et al., 1985; Sutton and Portier, 1989, 1991; Wooten and Elakovich, 1991). Elakovitch and Wooten (1989) tested 16 hydrophytes for their allelopathic potential using duckweed (*Lemna minor* L.) and lettuce seeds (*Lactuca sativa* L.) as bioassay materials. Among all the extracts, aqueous extracts of *Nymphaea odorata* leaves and petioles, *Nymphaea odorata* rhizomes, and entire plants of *Cabomba caroliniana* Gray., *Brasenia schreberi* Gmel., *Myriophyllum spicatum* Fernald, and *Vallisneria americanum* Michx., were most inhibitory as determined by both the bioassays. Jones (1993) reported that aqueous extracts of *Cobomba caroliana*, *Ceratophyllum demersum* L., *Myriophyllum aquaticum* L., *Myriophyllum spicatum*, *Nymphaea odorata*, *Potamogeton nodosus* Poir, *Sagittaria lancifolia* Pursh., *Pondtederia lanceolata* (Nutt.) Griseb., and *Vallisneria americana* affect the growth of *Hydrilla verticillata* (L.fil.) Royle. A review of the literature since 1970 reported 67 genera and 97 species of herbaceous vascular hydrophytes to have allelopathic potential (Elakovich and Wooten, 1995). However, no work has been reported on the allelopathic potential of the common aquatic plants associated with northern wild rice.

Lee (1987) observed dramatic growth enhancement of wild rice in the presence of *Potamogeton robinsii*. The mechanism of this growth enhancement is not known. It was assumed that *P. robinsii* increased phosphorus availability by

adding organic matter to clay sediments. However, sediment analysis could not detect such a high level of available phosphorus (Quayyum et al. unpublished data). Aqueous extracts of water hyacinth [*Eichornia crassipes* (Mart.) Solms] (Sircar and Kundu, 1959); *Carex alata* Torrey, and *Glyceria maxima* (Hartman) O. Holmb. (Szczepanska, 1977), and *Woolfia arrhiza* L., (Wolek, 1979) at low concentration were found to be stimulatory to several aquatic plants. Growth inhibitory properties of the aquatic plants as well as growth enhancement effects of *Potamogeton robbinsii* Oakes on wild rice suggest that allelopathy may be a possible mechanism.

The objective of the present study was to determine if the aquatic plants associated with northern wild rice and the lake sediments underneath them have allelopathic properties.

METHODS AND MATERIALS

Preparation of Water Extract of Plants. Nine plant species commonly associated with wild rice—*Eleocharis smallii, Scirpus acutus, Equisetum fluviatile, Nymphaea odorata, Nuphar variegatum, Sparganium fluctuans, Myriophyllum verticillatum, Potamogeton natans,* and *P. robbinsii*—were collected from Whitefish (Lat. 49°06′ N, Long. 90°09′W), Rice Stock (Lat. 48°13′N, Long. 90°00′W) and Chisamore (Lat. 49°20′N, Long. 89°44′W) lakes of northwestern Ontario. The freshly collected plants were separated into leaves and stems/rhizomes. One hundred grams of whole plants and their component samples were blended separately in 1 liter distilled water. The mixture was filtered through Whatman No. 1 filter paper using a vacuum pump. The pH of the extracts was determined immediately. The extracts were either assayed immediately or were stored at −15°C. The filtrates, considered as 100% (w/v) water extracts, were used for the bioassay experiments. We chose to make aqueous plant extracts on the assumption that the dead and decomposed plant materials release phytotoxic compounds in the environment that may also be transformed into other more toxic compounds (Patrick, 1971; Rice, 1984).

Preparation of Water Extracts of Lake Sediments. Lake sediment samples up to a depth of 20 cm from underneath the wild rice plants were collected from contiguous plots with and without the wild rice-associated plants present. Five samples were randomly collected from each of the paired plots with and without *Eleocharis smallii, Scirpus acutus, Nymphaea odorata, Nuphar variegatum, Myriophyllum verticillatum,* and *Potamogeton natans.* The 60 sediment samples were air dried at room temperature (25°C), ground, sieved (2 mm), and stored in paper bags. Two hundred fifty grams of air-dry sediment from each sample were soaked for 12 hr with 625 ml distilled deionized water at 4°C and then shaken for 2 hr at room temperature. The sediment extracts were filtered through Whatman No. 1 filter paper,

the pH determined, and extracts were stored at −15°C. The filtrates considered as 100% (w/v) sediment extracts, were used for the bioassay tests.

Total Phenolics. Phenolic compounds have been reported to be involved in allelopathic interactions (Rice, 1984). Since the additive or synergistic effects of different phenolics even at low concentrations can be influential in allelopathic interactions (Rasmussen and Einhellig, 1977; Einhellig and Rasmussen, 1978; Williams and Harland, 1982), the total phenolic contents of the aqueous extract of plants and their associated lake sediments were determined by the Folin-Chiocalteau reagent method (Swain and Hills, 1959).

Bioassay for Phytotoxicity of Extracts. The lettuce seedling bioassay was conducted with water extracts of different parts of individual plant species to determine their allelopathic potential. The pH of the extracts and control were adjusted with 2 N HCl and NaOH. Twenty lettuce seeds were placed in a disposable sterile Petri dish (9 cm diameter) lined with Whatman No. 3 filter paper. Four milliliters of the extract was added to each Petri dish. Control Petri dishes received 4 ml distilled deionized water. The Petri dishes were sealed with parafilm to prevent water loss and were stored in the dark at room temperature (22–24°C) for five days. Treatments were allotted in completely randomized design with five replications. Five days after incubation, percent germination and length of radicles (primary root) and plumules (primary shoot) were recorded.

Bioassay with Wild Rice Germinants. Aqueous extracts of the plants that exhibited strong growth inhibitory effects in the previous study were used to test their allelopathic potentials on wild rice. Ten uniform pregerminated wild rice seeds were placed in a disposable Petri dish (9 cm diameter) lined with one layer of Whatman No. 3 filter paper. Ten milliseconds of aqueous extract was added to each Petri dish. These were placed in a growth chamber at 14°C for one week, followed by another week at 16°C and a 16- hr photoperiod. The control germinants received 10 ml distilled deionized water. The treatments were replicated five times. Fifteen days after the incubation, root and shoot lengths were recorded. Root and shoot dry weights were determined by oven drying at 70°C for 48 hr.

Data Analysis. Analysis of variance was used to determine if the growth variables (root and shoot lengths and dry weights) of lettuce and wild rice seedlings were affected by the aqueous extracts of different plant samples. Significant differences among the means were determined by Tukey's HDS test.

RESULTS

Lettuce Seedling Bioassay. Aqueous extracts of all plant species inhibited lettuce seed germination compared to controls (Table 1). Ten water extracts of

the six plant species significantly reduced the primary root growth of lettuce seedlings (Table 1). Furthermore, there were marked differences between the extracts regarding the extent of root growth inhibition. The aqueous extracts of rhizomes of *Nymphaea odorata* and *Potamogeton natans* and the whole-plant extract of *Myriophyllum verticillatum* were most inhibitory, causing nearly 75% reduction in lettuce root length. Root growth of lettuce was not inhibited by the whole-plant extracts of *Equisetum fluviatile*, *Sparganium fluctuans*, and *Potamogeton robbinsii*. In contrast to root length, the shoot length of lettuce seedlings was stimulated by several plant extracts except the rhizome extract of *Nymphaea odorata* (Table 1), where shoot length was significantly reduced.

TABLE 1. EFFECT OF AQUEOUS EXTRACTS OF DIFFERENT PLANT SPECIES ASSOCIATED WITH WILD RICE PLANTS ON PERCENT GERMINATION AND ROOT AND SHOOT LENGTH OF LETTUCE[a]

Plant species and part(s)	Length		Germination (%)
	Root (mm)	Shoot (mm)	
Control	33.4 a	30.1 cd	100.0 a
Scirpus acutus			
Rhizome	14.2 cdef	28.7 cd	79.0 c
Shoot	22.1 cd	25.0 de	99.0 a
Eleocharis smallii			
Rhizome	25.9 bc	36.5 b	93.0 bc
Shoot	15.1 cdef	36.6 b	82.0 bc
Potamogeton natans			
Rhizome	7.7 fg	26.4 d	98.0 a
Leaves	17.8 cde	26.1 d	95.0 b
Nuphar variegatum			
Rhizome	18.7 cde	28.6 cd	95.0 b
Nymphaea odorata			
Rhizome	10.0 fg	19.8 e	96.0 a
Shoot	17.2 cde	35.3 b	97.0 a
Equisetum fluviatile			
Shoot	29.4 ab	46.0 a	91.0 bc
Myriophyllum verticillatum			
Shoot	8.6 fg	35.6 b	86.0 bc
Sparganium fluctuans			
Whole plant	29.7 a	29.3 cd	90.0 bc
Potamogeton robbinsii			
Whole plant	33.0 a	32.1 bc	90.0 bc

[a]Unlike letter(s) in a column indicate mean values are significantly different ($P = 0.05$) as determined by Tukey's HSD test.

Wild Rice Bioassay. Total root length of wild rice seedlings in rhizome extracts of *Scirpus acutus*, *Potamogeton natans*, *Nymphaea odorata*, and *Nuphar variegatum* and shoot extracts of *Eleocharis smallii* and *Myriophyllum verticillatum* was significantly less than that of the control (Table 2). Shoot length of wild rice was inhibited significantly only in the rhizome extract of *Nymphaea odorata* (Table 2).

Most treatments, except the whole-plant extract of *Potamogeton robbinsii*, caused a significant reduction of root length of wild rice (Table 2). Shoot length of wild rice was not affected significantly except by the rhizome extract of *Nymphea odorata*. Root dry weight was significantly reduced by all but the whole-plant extract of *Potamogeton robbinsii* (Table 2). Shoot dry weight of wild rice seedlings was significantly reduced by rhizome extracts of *Potamogeton natans* and shoot extracts of *Eleocharis smallii* (Table 2). Shoot growth was stimulated by the rhizome extracts of *Scirpus acutus* and *Nuphar variegatum* and the whole-plant extract of *Potamogeton robbinsii*. Shoot dry weight of wild rice in presence of the other extracts was not significantly different from control.

Effects of Lake Sediment Extracts. The root and shoot lengths and dry weights of root and shoot of wild rice seedlings did not differ significantly in the presence of water extracts of lake sediments with or without the wild rice-asso-

TABLE 2. EFFECTS OF AQUEOUS EXTRACTS OF AQUATIC PLANTS ON TOTAL ROOT AND SHOOT LENGTH AND ROOT AND SHOOT DRY WEIGHT OF WILD RICE SEEDLINGS[a]

Plant speices and part	Length (cm)		Weight (mg/plant)	
	Total root	Shoot	Root Dry	Shoot
Control	27.6 a	8.0 a	5.9 a	6.8 b
Scirpus acutus				
Rhizome	18.7 bcd	7.4 a	4.7 b	7.0 a
Eleocharis palustris				
Shoot	18.3 bcde	6.6 a	4.1 b	4.7 c
Potamogeton natans				
Rhizome	18.5 bcd	7.1 a	4.8 b	5.2 c
Myriophyllum verticillatum				
Whole plant	19.2 bcd	6.7 a	4.4 b	6.2 b
Nymphea odorata				
Rhizome	12.3 e	5.5 b	3.4 b	5.3 c
Potamogeton robbinsii				
Whole plant	26.6 a	7.7 a	6.1 a	7.1 a

[a]Unlike letters in a column indicate values are significantly different at $P = 0.05$ level as determined by Tukey's HDS test.

ciated plants. However, growth of wild rice seedlings was generally reduced in the extracts of sediments collected from underneath the wild rice plants.

Total Phenolics and pH. There was a large variation in total phenolic contents of the aqueous extracts of different plant species (Figure 1). The rhizome extract of *Nuphar variegatum* had the highest amount of total phenolics followed by the rhizome and whole-plant extracts of *Nymphaea odorata* and *Myriophyllum verticillatum*. The rhizome extract of *Scirpus acutus* had more phenolic content than the shoot extract of *Eleocharis smallii*. Among all extracts, the rhizome extract of *Potamogeton natans* had the lowest amount of total phenolics.

Total phenolic content of the lake sediment extracts was very low compared with that of the plant parts (Figure 2). However, the phenolic content of sediments collected from sites containing the rice-associated plants was always higher than that without the rice associated plants.

The pH of the water extract of the rice-associated plants varied from 4.2 to 6.4, while that of their sediments ranged between 5.7 and 7.0.

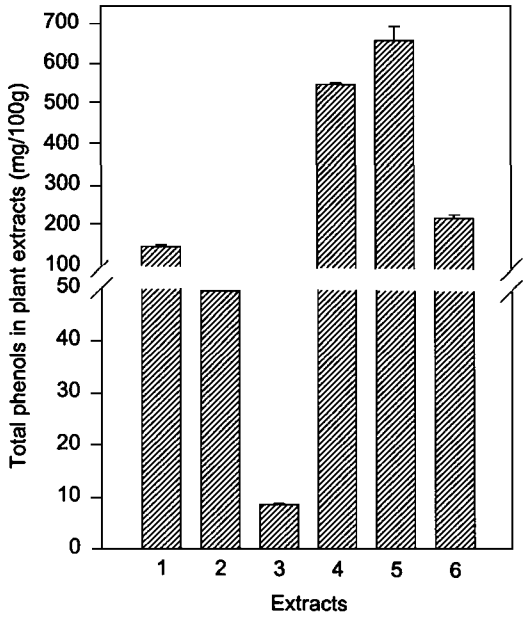

FIG. 1. Total phenolic contents of *Scirpus* rhizome extract (1), *Eleocharis* shoot extract (2), *Potamogeton* rhizome extract (3), *Nymphaea* rhizome extract (4), *Nuphar* rhizome extract (5) and *Myriophyllum* whole-plant extract (6).

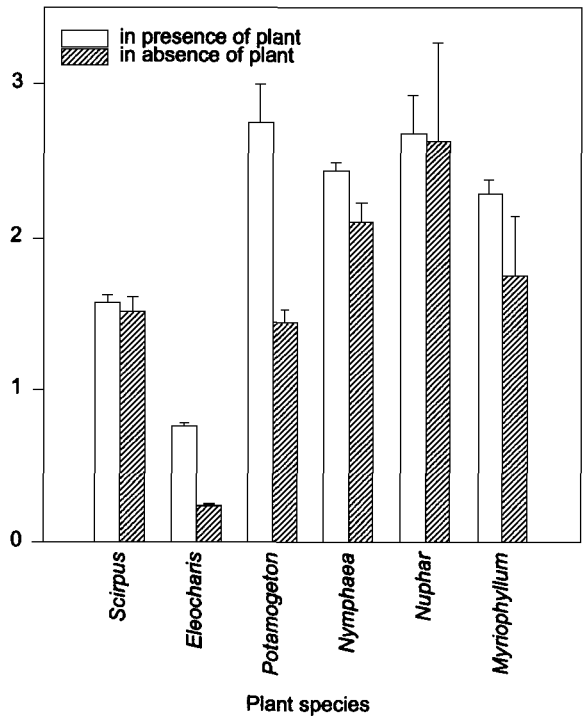

FIG. 2. Total phenolic content of lake sediments with and without wild rice associated plants.

DISCUSSION

Water extracts of some plants associated wild rice inhibited the primary root growth of lettuce as well as total root length of wild rice seedlings. Lettuce seedlings were more sensitive to the aqueous plant extracts than wild rice. Sensitivity of the bioassay material to the leachates or extracts of the aquatic species is important, but the high sensitivity of lettuce seedlings to plant extracts limits wider use as a standard bioassay material for evaluation of allelopathic effects and there is some doubt about the relevancy of using a nontarget species in understanding the interference mechanisms (Harper, 1977). Moreover, the response of a terrestrial plant may not be a true indicator of the interactions involved in aquatic plants. The importance of using target species as a bioassay material was suggested and used by several authors (Anderson, 1985; Inderjit and Dakshini, 1995). Einhellig et al. (1985) suggested duckweed (*Lemna minor*) as a suitable bioassay material for aquatic plants.

Inhibition of root growth by aqueous extracts was considered to be an acceptable variable for the evaluation of allelopathic interactions in plants (El-Ghazal and Reimer, 1986; Elakovich and Wooten, 1991; Zhu and Mallik, 1994). The reduction of root growth, both in lettuce and wild rice, indicates the presence of water-soluble growth inhibitory substances in aqueous extracts of some plant species. A number of phenolic compounds have been reported from allelopathic terrestrial plants (Rizvi and Rizvi, 1992; Zhu and Mallik, 1994; Inderjit and Dakshini, 1995; Inderjit, 1996). However, studies on allelopathic effects of aquatic plants are in their infancy (Gopal and Goel, 1993). Cheng and Reimer (1989) isolated gallic, vanillic, *p*-coumaric, and ferulic acids from American eelgrass (*Vallisneria americana*) and suggested that these phenolics are responsible for the alleged allelopathic properties of this weed. The rhizome of *Potamogeton natans* may contain other water-soluble allelochemicals such as organic acids, some water-soluble fatty acids, steroids, and sugars that are also considered as potential allelochemicals (Rice, 1984; Putnam and Tang, 1986). The stimulatory effects of some aqueous extracts on shoot growth of lettuce and wild rice suggest differential responses by roots and shoots to aqueous extracts. Both the inhibitory and stimulatory responses of plants are significant for assessing allelopathic properties (Rice, 1986). Chemicals with allelopathic potential are present in most of the plant parts. Whether these chemicals are released into the environment, particularly to the soil, and remain at bioactive concentrations for sufficient time to affect neighboring plants is a major consideration in the study of allelopathic interactions. Most investigators used aqueous plant extracts and lettuce and duck weed bioassays for evaluating the allelopathic potential of aquatic plants (Cheng and Reimer, 1988; Elakovich and Wooten, 1989, 1991). The growth inhibition of these bioassay plants by aqueous plant extracts does not, however, truly represent the allelopathic interactions in an ecosystem. Therefore, both aqueous plant extracts and the extracts from the associated sediment should be used with appropriate target species as a bioassay material for evaluating allelopathic interactions among plants.

In all the aqueous extracts of plants, the pH was above 4. Reynolds (1975) found that pH 2.6–6.4 had no effect on lettuce seed germination. We conclude, therefore, that growth inhibition of lettuce and wild rice by some extracts was not caused by the pH of the extracts.

No significant effect on the length and dry biomass of both roots and shoots of wild rice was obtained in presence of the aqueous extracts of lake sediments, with or without wild rice-associated plants. Thus the habitats containing the aquatic weeds do not seem to have any growth inhibitory effects on wild rice. The total phenolic contents of the sediments were much lower than those in the plant parts. These results are consistent with those obtained by investigators working on terrestrial systems (see Rice, 1984; and reviews by Inderjit and Dakshini, 1995; Inderjit, 1996). The lower phenolic contents of the lake sediments,

compared with the plant parts, may be due to many factors, for example: (1) a large part of the phenolic compounds coming from the plant parts may have dispersed in the lake water before reaching the lake sediments, (2) the phenolic compounds may have undergone anaerobic decomposition in the sediment (Dao, 1987), or (3) there may be some retention on the sediment colloids (Cheng, 1995; Blum and Shafer, 1988; Rice, 1984). The trend of slightly reduced root shoot growth of wild rice in presence of the lake sediment extracts indicates that there might be a small quantity of growth inhibitory compounds in the lake sediments (Dooris and Martin 1980).

It has been reported that the allelopathic effects are the result of additive or synergistic activity of allelochemicals (Einhellig and Rasmussen, 1978; Einhellig et al., 1982; Williamson, 1990). Under field conditions, these activities are high even at low concentrations (Einhellig et al., 1982; Williamson and Harland, 1982). Phenolic acids from living plants could accumulate in soil by root exudation, by decomposition of plant residues, or by microbial transformation into other toxic chemicals (Rice, 1984; Putnam, 1985). Furthermore, the accumulation of allelochemicals in soil varies with the seasons. Jalal and Read (1983) reported that the levels of aromatic and aliphatic acids in *Calluna* and spruce soil were higher in the summer months than at other times of the year. The inhibitory potential and amount of released phytotoxic from *Empetrum hermaphroditum* were highest during spring (Nilsson, 1992). Nilsson and Zackrisson (1992) also reported that phytotoxic substances accumulate in soil under conditions of ground ice. In the present study sediments associated with the plants were collected only once during the month of August. As it is the bioactive concentrations of phenolics in soil (which vary in different months of the year) that largely determines the visible allelopathic effects, it is necessary to determine the level of total phenolics in lake sediments at different months of the growing season. So far as we are aware, no previous study has reported on the possible phytotoxic property of plant phenolics of lake sediments containing aquatic plants.

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ALLELOPATHIC POTENTIAL OF AQUATIC PLANTS ASSOCIATED WITH WILD RICE: II. ISOLATION AND IDENTIFICATION OF ALLELOCHEMICALS

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Abstract—Aqueous extracts of rhizomes of *Scirpus acutus* and shoots of *Eleocharis smallii* were analyzed for the presence of phytotoxic compounds using ethyl acetate extraction. The organic fractions of the extract of *Scirpus* rhizomes contained lactic, succinic, fumaric, 2-hydroxysuccinic, 2-phenyl lactic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, protocatechuic, dehydroabietic, and ferulic acids; *p*-hydroxybenzyl alcohol, *p*-hydroxyphenyl ethanol and catechin. The extract of *Eleocharis* shoots contained 4-methoxy phenol, benzofuran, benzene acetic acid, 1-hydroxy-5-methyl acetophenone, and 1,3,4-dimethoxyphenol ethanone identified by GC-Mass spectroscopy. The potential allelopathic effect of these compounds on wild rice under field conditions was discussed.

Key Words—Allelochemicals, *Eleocharis smallii*, plant phenolics, *Scirpus acutus*, wild rice, *Zizania palustris*.

INTRODUCTION

Scirpus acutus Muhlenb. and *Eleocharis smallii* L. (hereafter referred to as *Scirpus* and *Eleocharis*, respectively) are common aquatic weeds associated with wild rice (*Zizania palustris* L.) in northwestern Ontario, Canada. These plants start their life cycles in lakes before the germination of wild rice and appear to attain a competitive advantage over wild rice. Aiken et al. (1988) reported that *Eleocharis* gradually displaces wild rice from shallow-water areas of the

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lakes. They also noted that the growth of wild rice was severely reduced when it occurs in presence of *Scirpus*. An earlier study showed that aqueous extracts of rhizomes of *Scirpus* and shoots of *Eleocharis* inhibited the root length of lettuce and wild rice seedlings (Quayyum et al., 1999). These extracts of *Eleocharis* and *Scirpus* were suspected to contain phytotoxic substances.

Phenolic acids are potential allelochemicals because of their high water solubility and plant growth inhibitory properties (Rice, 1984; Putnam and Tang, 1986; Zhu and Mallik, 1994; Inderjit, 1996). Phenolic acids are known to affect photosynthesis, protein synthesis, mineral uptake, chlorophyll synthesis, membrane permeability, and the water balance of plants (Rice, 1984). The phenolic compounds may be released from plants by leaching through fog drip or rain, by exudation from roots, or by degradation of plant parts (Hale and Orcutt, 1987). The two aquatic plants associated with wild rice were selected for characterization of their allelochemicals because of their wide distribution and known adverse effects on wild rice. Moreover, the narrow leaves of *Scirpus* should have little effect on competition for light or space; yet the weed has the ability to displace wild rice from lakes and river margins. Perhaps the decomposed organic matter of the plants releases phenolic substances into the rhizosphere of wild rice, inhibiting root growth.

The objective of the present study was to isolate and identify the water-soluble allelochemicals, particularly phenolics, from the extracts of *Scirpus* rhizomes and *Eleocharis* shoots, and to test their allelopathic potential. We intended to use wild rice as the bioassay material, but because of the short-term viability of the rice seeds, we were compelled to use lettuce seeds in this bioassay.

METHODS AND MATERIALS

Preparation of Water Extracts. *Scirpus* and *Eleocharis* plants were collected from Whitefish (Lat. 46°06′N, 90°09′W), Rice Stock (Lat. 48°13′N, Long. 90°00′W), and Chisamore (Lat. 49°20′N, Long. 89°44′W) lakes of northwestern Ontario. One hundred grams of rhizomes of *Scirpus* and shoots of *Eleocharis* were blended in 1 liter of distilled water. The mixture was filtered through Whatman number 1 filter paper using a vacuum pump.

Extraction of Phytotoxic Compounds by Fractionation. The aqueous extracts were fractionated according to Zhu and Mallik (1994) with some modification. A 250-ml water extract (fraction 1) was adjusted to pH 12 with 2 M NaOH. The alkaline medium ensures the conversion of phenolic acids into salts, which dissolve in water. The alkaline extract was centrifuged at 12,000*g* for 10 min. The supernatant was transferred to a separating funnel and was washed three times with 200 ml hexane to remove lipids (fraction 2). The pH of the resulting aqueous solution (fraction 3) was adjusted to pH 3 with 2 M HCl. The

acidified fraction was washed four times with 200 ml ethyl acetate. The ethyl acetate fraction containing phenolic compounds (fraction 4) was first dried over anhydrous sodium sulfate and then evaporated to dryness in a rotary evaporator at 39°C. The lower aqueous fraction containing sugars and inorganic compounds was separated (fraction 5) and retained.

Ethyl acetate organic fraction of water extracts of *Scirpus* and *Eleocharis* inhibited germination and the primary root growth of lettuce seedlings (Tables 1 and 2 below). These fractions were further analyzed to identify the growth inhibitory compounds.

Gas Chromatography–Mass Spectroscopy (GC-MS). The dried ethyl acetate fraction was dissolved in 4 ml methanol and passed through Whatman No. 1 filter paper. One milliliter of the methanol solution was transferred to a micro vial and evaporated to dryness under low air flow. The samples were silylated with *N,O*-bis(trimethylsilyl)trifluoracetamide (BSTFA) and then analyzed by GC high-resolution mass spectroscopy (GC-MS) (VG Auto Spec) at 50°C, isothermal for 2 min and then 80–350°C, for 5 min. The injection point temperature was 280°C. Spectral scans from total ion chromatograms were observed and matched using the National Institute for Standard and Technology (NIST) library compilation series.

Bioassay with Water Extract Fractions. Phytotoxicity of the various fractions of the water extract of *Scirpus* and *Eleocharis* was determined by lettuce seedling bioassay. One circle of Whatman No. 3 filter paper was placed in a Petri dish (9 cm diameter) and was wetted with 4 ml of the relevant fractions. After the solvent had evaporated from the hexane and ethyl acetate organic fractions, 4 ml deionized distilled water was added to each Petri dish. The control plates received only 4 ml deionized distilled water. Twenty lettuce seeds were placed on the filter paper of each Petri dish. The Petri dishes were sealed with parafilm to prevent water loss and were placed in a laboratory drawer at room temperature (22–25°C) for five days. Each extract was assayed by five replicate Petri dishes. Five days after incubation, the number of germinated seeds and the lengths of radicles (primary root) and plumules (primary shoot) were recorded.

Data Analysis. Data on root and shoot length and percent germination were analyzed by one-way analysis of variance (ANOVA), and significant differences among the means were determined by Tukey's HDS test. Arc-sine transformation was done on seed germination data prior to the ANOVA.

RESULTS

Isolation and Identification of Allelopathic Compounds. The lettuce seedling bioassay of the different fractions of *Scirpus* rhizome extract exhibited different degrees of root growth inhibition (Table 1). Fractions 1, 4, and

5 were more inhibitory than fractions 2 and 3. The reduced root growth in the hexane organic fraction (fraction 3) indicates that the partition of allelochemicals between organic and aqueous phases was incomplete. The shoot length of lettuce seedlings was not inhibited by any fractions of the water extracts. Germination of lettuce seeds was inhibited by different fractions of *Scirpus* water extracts. The ethyl acetate aqueous phase showed the highest inhibition followed by water extract, ethyl acetate organic, and hexane aqueous phases (Table 1).

Germination of lettuce seeds was inhibited by fractions 1, 2, and 4 of *Eleocharis* extracts (Table 2). The germination inhibition was highest in the aqueous extract. Large variation in germinations within replications was observed in hexane organic and ethyl acetate organic fractions. Lettuce seeds did not germinate in hexane aqueous and ethyl acetate aqueous fractions.

The primary root length of lettuce was inhibited in fraction 1 and 4 (Table 2). Again large variation in root length was observed in different replications of fraction 4. Fraction 2 did not inhibit root length. Shoot length was inhibited only in fraction 4 (Table 2).

When evaluated by GC-MS, the ethyl acetate organic fraction of *Scirpus* revealed the presence of more than 40 compounds (Figure 1). Among them, 14 peaks were identified as lactic acid, succinic acid, fumaric acid, 2-hydroxysuccinic acid, *p*-hydroxybenzyl alcohol, *p*-hydroxyphenyl ethanol, 2-phenyl lactic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid (*p*-hydroxybenzoic acid), protocatechuic acid, ferulic acid, dehydroabietic acid, and a dye, catechin, by comparison with the mass spectral data base. A peak with a retention time of 26.13 was identified as a phthalate and other peaks were common glass contaminants.

The ethyl acetate aqueous fraction of the *Scirpus* extract revealed the presence of several sugar moieties when evaluated by GC-MS (data not shown).

TABLE 1. EFFECT OF DIFFERENT FRACTIONS OF *Scirpus* RHIZOME EXTRACT ON GERMINATION AND ROOT AND SHOOT LENGHTS OF LETTUCE SEEDLINGS[a]

Fractions	Germination (%)	Root length (mm)	Shoot length (mm)
Control	96.0 ± 3.6 a	29.0 a	28.5 a
1. Water extract	56.0 ± 12.8 b	15.7 d	30.0 a
2. Hexane organic phase	88.0 ± 9.4 a	23.8 b	28.2 a
3. Hexane aqueous phase	54.0 ± 14.2 b	18.2 c	27.9 a
4. Ethyl acetate organic phase	48.0 ± 20.5 b	17.0 cd	28.2 a
5. Ethyl acetate aqueous phase	18.4 ± 18.3 c	16.5 cd	26.9 a

[a]In a column, means followed by the same letter(s) are not significantly different at 0.01 percent level by Tukey's HDS test.

TABLE 2. EFFECT OF DIFFERENT FRACTIONS OF *Eleocharis* SHOOT EXTRACT ON GERMINATION AND ROOT AND SHOOT LENGTH OF LETTUCE SEEDLINGS[a]

Fraction	Germination (%)	Root length (mm)	Shoot length (mm)
Control	98.0 ± 4.5	41.0 ± 2.8	28.6 ± 1.4
1. Water extract	26.0 ± 11.4	12.1 ± 0.8	26.4 ± 0.6
2. Hexane organic phase	72.0 ± 21.7	38.7 ± 5.6	29.0 ± 3.4
3. Hexane aqueous phase	0[b]		
4. Ethyl acetate organic phase	40.0 ± 31.6	9.1 ± 8.3	7.3 ± 6.7
5. Ethyl acetate aqueous phase	0[b]		

[a]Values are means ± standard deviation.
[b]No germination was obtained in aqueous phase of hexane and ethyl acetate.

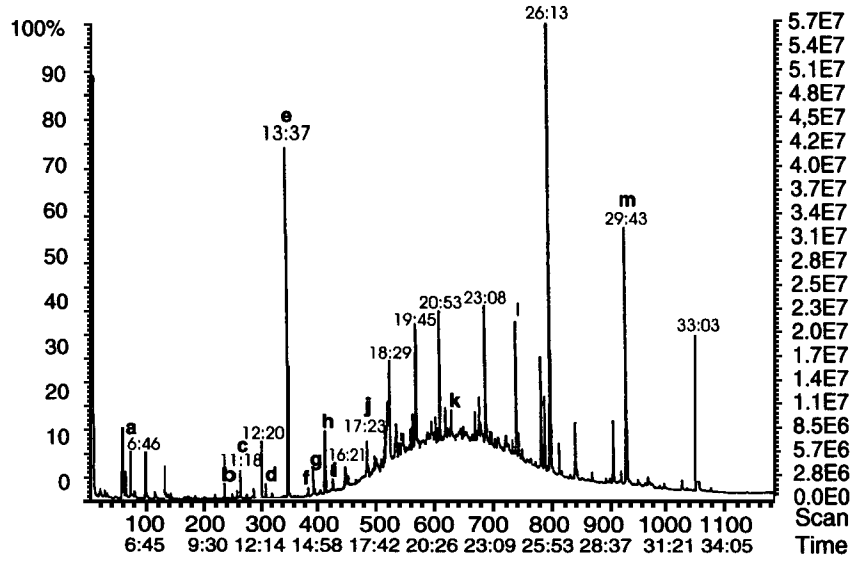

FIG. 1. Total ion current chromatogram of GC-MS of ethyl acetate organic fraction of *Scirpus* rhizome extract: a, lactic acid; b, succinic acid; c, fumaric acid; d, *p*-hydroxybenzyl alcohol; e, 2-hydroxysuccinic acid; f, *p*-hydroxyphenyl ethanol; g, 2-phenyl lactic acid, h, *m*-hydroxybenzoic acid; i, *p*-hydroxybenzoic acid; j, protocatechuic acid; k, ferulic acid; l, dehydroabietic acid; and m, catechin.

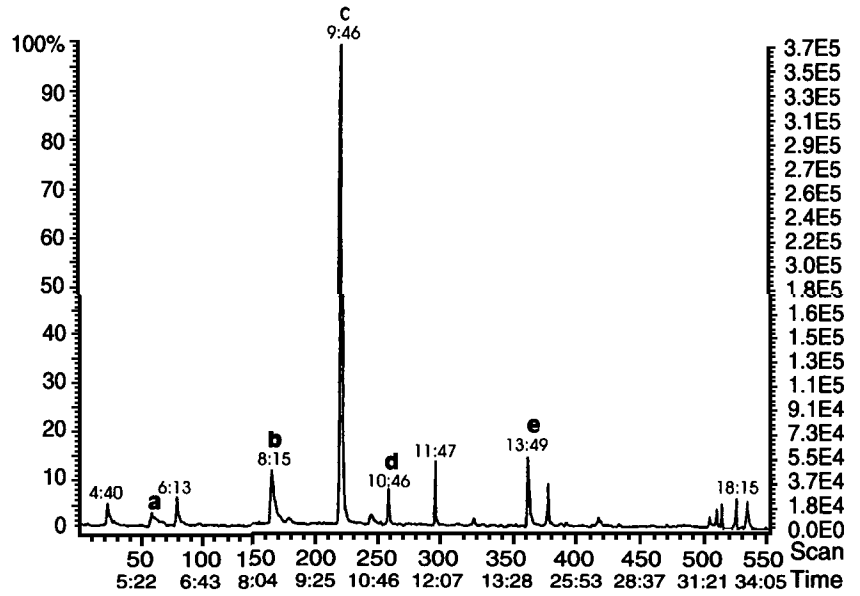

FIG. 2. Total ion current chromatogram of ethyl acetate organic fraction of *Eleocharis* shoot extract: a, 4-methoxy phenol; b, benzofuran; c, 1-hydroxy-5-methyl acetophenone; d, benzene acetic acid; and e, 1,3,4-dimethoxyphenyl ethanone.

The ethyl acetate organic fraction of *Eleocharis* extract showed the presence of more than 14 compounds when analyzed by GC-MS (Figure 2). Five peaks were identified as 4-methoxy phenol, benzofuran, benzene acetic acid, 1-hydroxy-5-methyl acetophenone, and 1,3,4-dimethoxy phenyl ethanone; four peaks were sugar moieties, and others were contaminants. The ethyl acetate aqueous fraction of *Eleocharis* revealed several peaks and most of them were sugar moieties identified by GC-MS (data not shown).

DISCUSSION

Fifteen compounds were identified from the ethyl acetate organic fractions of *Scirpus* rhizome extract (Figure 1). Fumaric acid is a well known microbial toxin that also exudes from seeds of *Pinus resinosa* and inhibits the growth of *Pythium afertile* (Mirocha et al., 1966). The benzoic acid derivatives, *m*-hydroxybenzoic and *p*-hydroxybenzoic acids have been isolated from fresh and decomposed plant parts and soil as potential allelochemicals. The latter compound is one of the most common benzoic acid derivatives involved in allelopathy (Rice, 1984). It has been identified from branch leachate of *Arctostaphylos glandulosa*

(Chou and Muller, 1972), subtropical grasses (Chou and Young, 1975), decomposing rye and corn residues in soil (Chou and Patrick, 1976), forest soils (Lodhi, 1976), and leaf leachate of *Kalmia angustifolia* (Zhu and Mallik, 1994). Protocatechuic acid has also been reported as an allelopathic agent. In plants, it originates directly from dehydroshikimic acid (Neish, 1964). It has been isolated from leachate of *Arctostaphylos glandulosa*, water extracts of *Erica* spp. and its associated soil (Carballeira, 1980), and from *Leucaena leucocephala* (Chou and Kuo, 1986). Ferulic acid is a cinnamic acid derivative, and it has been isolated from aqueous extracts of different plant parts, crop residues, and soil under allelopathic plants (Rice, 1984; Chou and Kuo, 1986; Zhu and Mallik, 1994). Although these compounds were isolated and identified from *Scirpus* rhizomes in the present study, their allelopathic effects on wild rice were not tested. No report is available on the allelopathic properties of other compounds identified from the aqueous extract of *Scirpus* rhizomes.

Five compounds—4-methoxy phenol, benzofuran, benzene acetic acid, 1-hydroxy-5-methyl acetophenone, and 1,3,4-dimethoxyphenyl ethanone—were identified from shoot extracts of *Eleocharis*. There is no previous report of these compounds being allelochemicals.

Ethyl acetate aqueous fractions of *Scirpus* rhizome extract and that of *Eleocharis* shoot contained only sugar moieties. At low concentration, sugars generally do not inhibit germination and growth of plants, but at higher concentrations they may affect plant growth by increasing the osmotic potential of the growth medium. High osmotic concentrations induce stress in plants, primarily by causing dehydration of plant material (Slayter, 1967; Waring and Cleary, 1967). However, we believe that sugar concentrations of these aqueous fractions were not high enough to induce osmotic stress. The exact mechanism by which these two extracts inhibited the growth of lettuce seedlings can not be explained from this study.

Since these plants are perennials, they may maintain a sufficient supply of phytotoxic compounds in lake sediments through exudation and/or decomposition of the plant parts that might affect the growth of wild rice seedlings. Further studies will be necessary on bioactive concentration of these compounds in lake sediments underneath the plants to understand their roles in allelopathic interaction.

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IDENTIFICATION OF HOST-RELATED VOLATILES ATTRACTIVE TO PINEAPPLE BEETLE *Carpophilus humeralis*

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Abstract—Volatiles collected from oranges fed upon by *Carpophilus humeralis* of either sex were consistently more attractive than volatiles from beetle-free oranges in wind-tunnel bioassays. Three compounds were identified as attractants from this system: 4-ethyl-2-methoxyphenol (**1**), 2,5-diisopropylpyrazine (**2**) (a new natural product), and 2-phenylethanol (**3**). Identifications were confirmed with synthetic compounds that had matching chromatographic and spectral properties. Compounds **1**, **2**, and **3** had only slight activity alone, but were highly synergistic with each other and with propyl acetate (PA), a fruity ester that is mildly attractive to *Carpophilus* beetles. Compound **2** was the most active in the wind tunnel; its threshold dose was 0.5 ng when PA was present. The structural specificity for these compounds was high. Twelve phenol analogs of **1** were tested, but only one of these, 2-methoxyphenol, was more attractive than the control. Similarly, the analogs of **2**, 2-isopropylpyrazine and 2,6-diisopropylpyrazine, were completely inactive. In the field, a combination of **1**, **2**, and **3** was not attractive by itself, but it strongly synergized attraction to fermentation volatiles, *Carpophilus* pheromones, or both. Compounds **1**, **2**, and **3** apparently have a microbial origin because all three

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were detected when the host fruit was pineapples instead of oranges, because they could occur in the absence of beetles, and because autoclaved pineapple began to produce the compounds after inoculation from an attractive piece of fruit. The study demonstrated that host location for this generalist species can be far more complex than responding simply to the bouquet of low-molecular-weight volatiles normally associated with fermentation.

Key Words—*Carpophilus humeralis*, 4-ethyl-2-methoxyphenol, 2,5-diisopropylpyrazine, 2-phenylethanol, microbe produced attractants, synergism, structure–activity relationship, field trials.

INTRODUCTION

The pineapple beetle *Carpophilus humeralis* (F.) (Coleoptera: Nitidulidae) occurs in warm-weather regions throughout the world (Connell, 1981), is a pest of numerous fruits and vegetables (Gillogly, 1962), and is particularly attracted to fermenting plant material, often forming large aggregations on its host (Schmidt, 1935). *C. humeralis* can damage crops directly through feeding or indirectly by vectoring rot-causing microorganisms (Chang and Jensen, 1974). Economic losses attributed to this beetle have occurred in pineapples (Gallardo-Covas, 1984), dates (Kehat et al., 1983), and sugarcane (Chang and Jensen, 1974).

The need to control *Carpophilus* species with less reliance on insecticides has led to research on attractants for these beetles. Male-produced aggregation pheromones have been identified for nine *Carpophilus* species and are highly attractive in the field, especially in combination with fermenting food materials (Bartelt, 1997). It is known that *C. humeralis* responds to the pheromones of other *Carpophilus* species. For example, the pheromone of *C. freemani* Dobson attracted *C. humeralis* in South Carolina (Bartelt et al., 1995). The pheromone for *C. hemipterus* (L.) also attracted *C. humeralis* in both Israel (Blumberg et al., 1993) and Australia (James et al., 1994), but no pheromone is yet known for *C. humeralis*. Previous studies on attraction of *Carpophilus* beetles to hosts focused on fermentation volatiles such as acetaldehyde, ethanol, and other small polar compounds (Smilanick et al., 1978; Phelan and Lin, 1991; Lin and Phelan, 1991). *C. humeralis* is also highly attracted to fermenting microbial cultures and to synthetic blends of compounds identified from these (Nout and Bartelt, 1998; Bartelt and Zilkowski, 1998).

A pheromone was sought from *C. humeralis* using procedures that were successful with other *Carpophilus* species (e.g., with artificial diet as a food source) (Bartelt et al., 1993), but this effort failed (unpublished data). The work was repeated with natural host fruit (oranges) in a further effort to demonstrate pheromone existence. Sex-specific attractants were still not found but, surprisingly, the orange pieces fed on by beetles of either sex became highly attractive relative to the control orange pieces. This report describes the identification and

bioassay of the compounds primarily responsible for this attraction and explores their origins.

METHODS AND MATERIALS

Beetle Cultures. The culture of *C. humeralis* originated from a date garden near Oasis, California. Beetle rearing was as described by Dowd and Weber (1991), using a modified diet (Bartelt et al., 1993). Insects from the culture were used for volatile collections and for wind tunnel bioassays.

Volatile Collections. Volatiles were collected by using traps of 7-cm × 0.5-cm-ID glass tubing, sealed at one end with brass screen (100 mesh), that contained 0.5 cm of Super-Q porous polymer (Alltech, Deerfield, Illinois). Traps were attached to flasks containing either male *C. humeralis* and food, female *C. humeralis* and food, or food only, using procedures similar to those employed for isolating pheromones from *C. hemipterus* (Bartelt et al., 1990). In the initial studies, 250 g of sliced whole Valencia oranges (peel and segments) were placed into each volatile collector (1-liter filtering flask), which also contained 200 g of dried hexane-washed sand to absorb excess moisture. The flasks with beetles were set up with about 250 individuals, 2–5 days old. Airflow through the flasks was 50 ml/min. The flasks were maintained in an incubator at 27°C and a light–dark cycle of 14 : 10 hr. Fresh fruit was added to the flasks once a week. Although the oranges in flasks containing beetles became visibly moldy and decomposed within a few days, the beetles remained active and apparently healthy. (*C. humeralis* uses fallen, moldy oranges as a host resource in the field.) Orange pieces in control flasks had far less mold and generally remained intact. The flasks were maintained this way for periods of three to six weeks. Then the materials and insects were discarded and clean flasks were set up with new oranges and beetles. This procedure was repeated five times to provide a constant supply of volatiles for analysis.

Super-Q filters were removed every two or three days and back-flushed with 500 μl hexane to remove trapped volatiles. Filters were then put back into place after removing residual solvent by vacuum. Collected samples were stored at −5°C until needed. Collection of volatiles on Super-Q is biased toward compounds of higher molecular weight and is inefficient for small fermentation compounds such as acetaldehyde and ethanol, which are typically host-related attractants for *Carpophilus* beetles.

Most volatile collections were bioassayed to monitor changes in the attractiveness of flask contents over time. For each of the three treatments—males with oranges, females with oranges, or oranges only—collections with similar levels of activity were pooled for subsequent chemical analysis. The isolation and eventual identification of all the attractive compounds reported did not occur

from any one pooled collection. Isolation techniques were refined with subsequent collections and the final methods used are described below.

After the work with the oranges was complete, volatile collections were continued using fresh pineapple pieces as the food source. This was done to investigate the origin of the identified attractants. Eventually, volatile collections were also made from autoclaved pineapple pieces (30 minutes at 115°C), some of which were inoculated with material from attractive flasks.

Wind-Tunnel Bioassays. The attractiveness of volatile collections and subsequent chromatographic fractions for *C. humeralis* was assessed by wind-tunnel bioassay [previously described by Bartelt et al. (1990)]. A modification was the use of a small space heater, mounted near the air inlet, to warm the incoming air to about 30°C. Ambient relative humidity in the wind tunnel was generally 20–30%.

Briefly, approximately 500–2000 *C. humeralis* adults (sexes not determined), 1–2 weeks old, were placed into the wind tunnel, 2–3 hr after the start of the light cycle. Generally within 3 hr, beetles would begin to fly about, and bioassays would start. Insects would remain responsive for periods of up to 4 hr. Treatment solutions or hexane (blank control) were applied to 7-cm filter paper disks folded into quarters and suspended at the upwind end of the wind tunnel. Samples for bioassay were usually 10 μl. (For the periodic collections, this represented about 2% of the material accumulated per flask per collection). These baits were always tested in pairs, hung side by side, and separated from one another by 0.3 m. Tests lasted 2 min, and the total number of insects that landed on each treatment was recorded. There was a 2- to 4-min pause between tests while the next paper disks were prepared. Treatment locations were alternated for subsequent replications to avoid wind-tunnel position effects. Up to 35 tests could be performed each bioassay day, although 10–20 was typical.

Unless otherwise stated, propyl acetate (PA) was added to each treatment and control (~20 μl of 10% PA in mineral oil, applied to one side of the filter paper circle away from the test solution). As with other *Carpophilus* species (Dowd and Bartelt, 1991), *C. humeralis* responded to blends of small esters and fermentation volatiles; one of these, PA, was only slightly attractive by itself but strongly magnified the effects of many other compounds.

When comparisons among three or more treatments were desired, all possible two-way tests were run using a balanced incomplete block design. For statistical analysis, log $(X + 1)$ transformations of the data were employed to stabilize variances. Analysis of balanced incomplete block designs was by the method of Yates (1940). Each 2-min test was considered to be a block. Statistical differences among log transformed means were determined by the honestly significant difference (HSD) method. Further experimental details are given in the Results section.

The experiments involving analogs of attractant chemicals contained more

treatments than could be compared in one bioassay day. Therefore, each treatment was compared to the PA control and to an appropriate standard compound in a small, balanced, incomplete block design. Then the relative activity of each treatment was calculated as an index of activity, which expressed the attraction of each test compound as a percentage of the standard compound (corrected for the control response) according to the following equation:

$$\text{Index of activity} = \frac{(\text{test compound} - \text{PA control})}{(\text{standard compound} - \text{PA control})} \times 100$$

An index of activity of 0 indicates activity at the control level, and 100 indicates activity as for the standard compound.

Chromatography. Volatile collections and derived chromatographic fractions were analyzed by a Hewlett-Packard 5890 Series II gas chromatograph (GC) interfaced to a Hewlett-Packard 3396A integrator. The GC was equipped with a flame ionization detector and a Hewlett-Packard 7673 autosampler. A 15-m × 0.25-mm-ID capillary column (DB-1, with 1.0-μm film thickness, J&W Scientific, Folsom, California) was employed with 2-μl sample injections done in splitless mode. The oven temperature was programmed from 50°C to 270°C at 10°C/min; the injector temperature was 220°C, and the detector temperature was 270°C.

High-performance liquid chromatography (HPLC) was conducted on pooled volatile collections using a Spectra-Physics model SP8700 pump and a Rainin Microsorb silica column (4.6 mm ID × 25 cm long). An LDC/Milton Roy SpectroMonitor D UV variable wavelength detector set at 254 nm was used to monitor the separations, but all fractions were also analyzed by GC. Initial HPLC separation of whole volatile material was done using an elution gradient of 100% hexane for 5 min, followed by increasing ether content by 1% each minute for 30 min; ether content was then increased to 100% (7% per minute) over the final 10 min. Injection volume was 100–200 μl, repeated until the volatile material (up to 2 ml) from each source (males, females, and oranges) was fractionated. Flow rate was 1 ml/min and effluent was collected as 1-ml fractions. Like fractions from the replicate runs were combined.

One of the active fractions from the initial HPLC separation (fraction 16, see below) was subjected to a second HPLC gradient. This second HPLC elution gradient was 3% ether in hexane initially, increasing to 20% ether in hexane over 30 min.

Preparative GC fractionation of some attractive mixtures was done using a Varian 3700 GC equipped with a flame ionization detector, 1 : 1 effluent splitter, and a thermal gradient collector (Brownlee and Silverstein, 1968). Cooling was by a solution of Dry Ice in ethanol. A Linear 1200 recorder was used to

visualize chromatograms. The oven temperature was programmed from 50°C to 250°C at 10°C/min; the injector temperature was 200°C, and the detector temperature was 270°C. A 15-m DB-1 capillary column (previously described) was used, with 300 μl of sample concentrated to 5 μl and injected in splitless mode. GC effluent condensed in glass capillary tubes (1.1–1.2 mm ID, 30 cm long), which were exchanged at intervals to capture successive fractions. Fractions were rinsed from the tubes into vials with 100 μl of hexane.

Spectroscopy. Electron-impact mass spectra of attractive compounds were obtained on a Hewlett-Packard 5970 mass selective detector. Sample introduction was through a Hewlett-Packard 5890A gas chromatograph fitted with a 15-m DB-1 capillary column (previously described), using an oven temperature program similar to that described above. The high-resolution mass spectrum (sample injection through GC inlet) was provided by the University of Illinois Mass Spectrometry Laboratory. NMR proton and carbon spectra for synthesized pyrazines were obtained on a Bruker 300-MHz instrument. Samples were dissolved in deuterochloroform, and shifts were calculated relative to tetramethylsilane.

Base Extraction. Base extraction was used to separate acidic compounds from neutral compounds in one HPLC fraction (fraction 17, see below). A 500-μl volume was concentrated to 100 μl under nitrogen. About 100 μl of 10% aqueous KOH was added to the vial and stirred with a vortex mixer, then the upper (hexane) layer was removed using a Pasteur pipet. The KOH extract was then washed twice with 100 μl of hexane and the hexane washes were combined. HCl (5%) was then added dropwise to the KOH solution until it turned acidic (as indicated by pH paper). The acidified mixture was washed (three times) with 250 μl of hexane; the hexane layers were removed by pipet and combined. The hexane extract of the acidified mixture was concentrated under nitrogen and analyzed by GC and wind tunnel bioassay.

Synthesis of Diisopropylpyrazines. Synthesis of 2,5- and 2,6-diisopropylpyrazines (**2** and **17**, respectively) was done following the method of Seeman et al. (1992). This quick and simple synthesis involved the reaction of acetone with 2-isopropylpyrazine and sodium metal in absolute diethyl ether. The acetone and sodium metal formed a ketyl with which the 2-isopropylpyrazine reacted to produce both 2,5- and 2,6-diisopropylpyrazine.

A second synthesis produced only the 2,5 isomer of diisopropylpyrazine and allowed the products of the first synthesis to be unambiguously identified. An enolate formed from ethyl acetate at −78°C in tetrahydrofuran, using lithium diisopropylamide as the base, was condensed with isobutyryl chloride to form ethyl 4-methyl-3-keto-pentanoate (March, 1985, p. 436). This keto ester was hydrolyzed to the sodium salt by stirring with 5% NaOH at room temperature. The salt was then acidified with acetic acid and treated with sodium nitrite as described by Ferris et al. (1968) to afford 1-hydroxyimino-3-methyl-2-butanone. [The carbonyls at the 1 and 3 positions of the free acid directed the nitrite attack

specifically to the 2 carbon, and the carboxyl (1) carbon was then spontaneously eliminated as CO_2, giving the above keto oxime (Krems and Spoerri, 1947).] The keto oxime was reduced with aluminum amalgam (Ferris et al., 1968), and the free keto amine condensed spontaneously in head-to-tail fashion to form 1,4-dihydro-2,5-diisopropylpyrazine. Oxidation to the final product was with mercuric chloride (Krems and Spoerri, 1947). All reactions were monitored by GC and MS. Product was purified by distillation (Kugelrohr) and column chromatography on silica (elution with CH_2Cl_2).

The products from the first synthesis were purified by HPLC using 12% ether in hexane as the solvent system, run isocratically at 1 ml/min. Complete separation of the two compounds was achieved when 0.2 mg of the product mixture was injected. Purity of the recovered isomers was greater than 90% for 2,5-diisopropylpyrazine, which eluted 7.5–8.3 ml after injection (individual impurities less than 4%), and greater than 97% for 2,6-diisopropylpyrazine, which eluted 8.6–10.5 ml after injection (individual impurities less than 1%). The GC retention times for 2,6- and 2,5-diisopropylpyrazines were 8.91 min and 9.33 min, respectively. Spectral data were obtained for the diisopropylpyrazines. For 2,5-diisopropylpyrazine, the mass spectrum is presented in Results; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.377 (s, 2H), 3.069 (sept., J = 6.9 Hz, 2H), 1.307 (d, J = 6.9 Hz, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.228 (CH_3), 33.545 (alkyl CH), 141.855 (ring CH), 159.312 (ring C). For 2,6-diisopropylpyrazine: EI-MS [m/z (%)]: 164 (M^+, 28), 149 (100), 136 (66), 121 (7), 107 (4), 94 (4), 80 (5), 67 (17), 53 (30), and 41 (40); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.260, (s, 2H), 3.051 (sept., J = 6.9 Hz, 2H), 1.307 (d, J = 6.9 Hz 12 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.184 (CH_3), 34.038 (alkyl CH), 139.866 (ring CH), 160.999 (ring C).

Additional Chemicals. 4-Ethyl-2-methoxyphenol (**1**) (98%), 2-methoxy-4-methylphenol (**12**) (98%), 2-methoxy-5-methylphenol (**13**) (98%), 3-methoxyphenol (**14**) (97%), and 4-methoxyphenol (**15**) (98%) were purchased from Lancaster Synthesis Inc., Windham, New Hampshire. 2-Phenylethanol (**3**) (99%), 2-methoxyphenol (**4**) (98%), 4-allyl-2-methoxyphenol (**6**) (99%), 4-allyl-1,2-dimethoxybenzene (**7**) (99%), 4-ethylphenol (**8**) (99%), and anisole (**10**) (99%), were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Phenol (**11**) (99%) was purchased from J. T. Baker Chemical Co., Phillipsburg, New Jersey. 2-Isopropylpyrazine (**16**) (99%) was obtained from Pyrazine Specialties Inc., Atlanta, Georgia. 4-Ethyl-1,2-dimethoxybenzene (**5**) was made from **1** by the procedures of Johnstone and Rose (1979) and 3-methoxy-1-ethylbenzene (**9**) was similarly made from 3-ethylphenol (Aldrich).

Field Bioassays. Field testing of **1**, **2**, and **3** was done at sites where *C. humeralis* populations were known to exist. Tests were conducted in field corn near Blackwell, South Carolina; in a date palm orchard near Bet Dagan, Israel; and in a Valencia orange grove near Leeton, New South Wales, Australia.

Field tests were performed in randomized complete blocks with two replications at each location, either done simultaneously (South Carolina and Australia) or consecutively (Israel). There were six treatments: (1) control; (2) a rubber septum loaded with 500 μg each of **1**, **2**, and **3**; (3) food-type attractant [bread dough (South Carolina and Australia) or grapefruit (Israel)]; (4) **1**, **2**, **3**, and food; (5) food and a septum loaded with synthetic pheromone (500 μg) of either *C. hemipterus* (Australia and Israel) or *C. freemani* (South Carolina); and (6) **1**, **2**, **3**, food, and pheromone. The composition of pheromone septa was as described previously (Bartelt et al., 1995).

Wind-oriented pipe traps (Dowd et al., 1992) were spaced 10-m apart and hung 0.3 m above ground level on either tree trunks or branches (for tests in corn, traps were placed in trees adjacent to the field). In South Carolina and Australia, traps were serviced weekly (for a total of eight weeks in South Carolina, four weeks in Australia) to collect captured insects, replace food coattractant, and change the pheromone septa. Septa containing **1**, **2**, and **3** were changed every two weeks. In Israel, captured insects were collected twice a week and all baits were replaced every week (4 weeks total). Total trap catches for each treatment, replicate, and test site were analyzed. The log $(X + 1)$ transformation was used to stabilize variance. Linear contrasts were used to test for effects of **1**, **2**, **3**, and pheromones.

RESULTS

Initial Bioassays. Volatiles were collected two days after the treatments were set up. They attracted few beetles in the wind tunnel (usually <10), and the treatments with beetles were not different from the controls with oranges alone. By the second volatile collection (two days later), samples from *C. humeralis* of either sex on oranges were consistently more attractive than collections from oranges only, and this activity remained consistent with subsequent collections (in some cases for periods up to 6 weeks). Once this difference became established, the collections for the individual treatments were pooled for chemical analysis. The pooled collections for the five separate setups had mean bioassay responses of 47.1 landings for males on oranges, 63.9 landings for females on oranges, and 4.4 landings for oranges only.

Analysis of the whole volatile collections (male, female, and orange only) by GC revealed very complex profiles (e.g., Figure 1A). After separation of the beetle-derived volatile collections by HPLC, only three fractions (13, 16, and 17) had clear activity (Figure 1B). The male- and female-derived fractions gave similar bioassay results, but no fractions from oranges without beetles were more attractive than the PA control. GC profiles of the three active fractions are shown in Figure 1C–E. (The examples shown are from volatile collections of

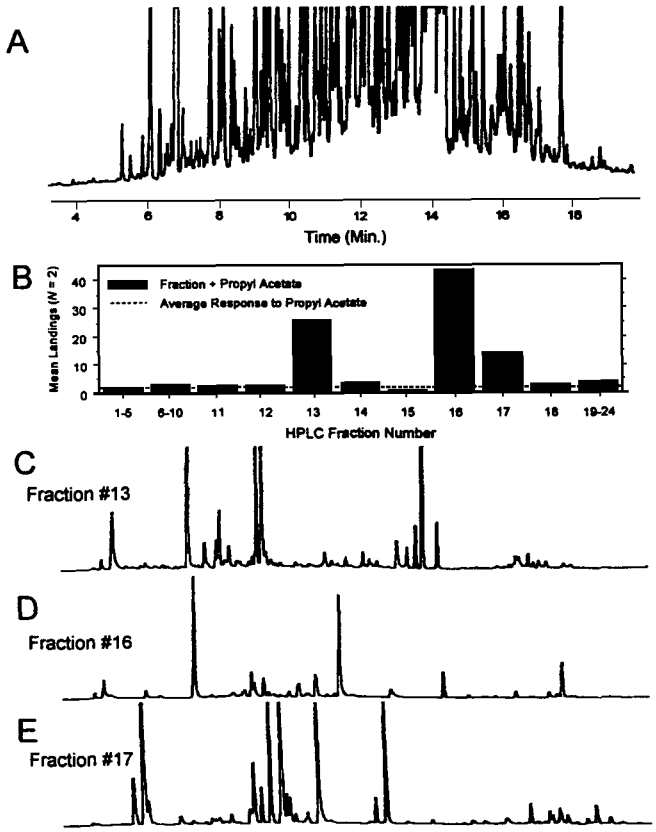

FIG. 1. (A) Gas chromatogram of an attractive volatile collection from male beetles on oranges prior to separation by HPLC. (B) Wind-tunnel bioassay results for fractions collected from the HPLC gradient of the whole volatile collection (fractions 1–5, 6–10, and 19–24 combined due to the small amount of material present in each). (C–E) Gas chromatograms of attractive fractions 13, 16, and 17, respectively. Chromatograms represent about 1% of the amount of material in a two-day volatile collection.

males on oranges but those from females were very similar). Hydrocarbon compounds, such as the pheromones of the other *Carpophilus* species, would have been present in fractions 1–5, but these fractions were unattractive and no male-specific compounds were detected by GC.

Identification of 4-Ethyl-2-methoxyphenol. The GC profiles of HPLC fraction 17 from males (and females) contained a peak (retention time 10.66 min) that was absent from the orange-derived fraction (Figure 2A, compound **1**). GC-MS and subsequent library search suggested that compound **1** was 4-ethyl-2-

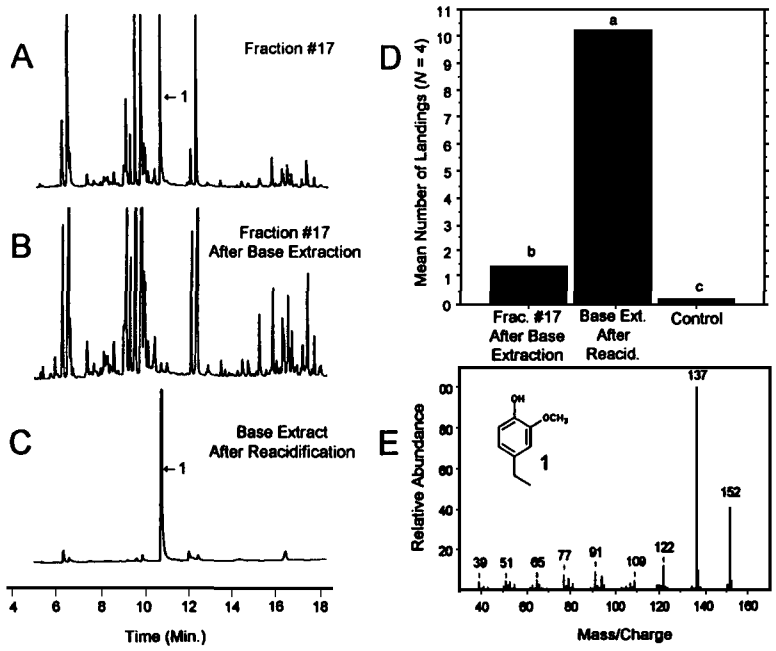

FIG. 2. (A) Gas chromatogram of active HPLC fraction 17. Compound **1** was found in both attractive male and female collections but was absent in unattractive orange collections. (B) Gas chromatogram of fraction 17 after extraction with KOH. (C) Gas chromatogram of base-extracted material from fraction 17 after reacidification with HCl. (D) Wind-tunnel bioassay results of base-extracted fraction 17 and the reacidified material. (E) Structure and mass spectrum of 4-ethyl-2-methoxyphenol (**1**).

methoxyphenol. Extraction of fraction 17 with KOH removed nearly all of compound **1** (Figure 2B), and **1** was readily recovered by reacidification of the base extract followed by extraction with hexane (Figure 2C). Wind-tunnel bioassays showed that the acidic material, containing predominantly compound **1**, was significantly more attractive than the nonacidic material (Figure 2D). Compound **1** from fraction 17 was identical to authentic 4-ethyl-2-methoxyphenol by both GC retention and mass spectrum (Figure 2E).

Identification of 2,5-Diisopropylpyrazine. Comparison of GC profiles of the active HPLC fraction 16 from males or females on oranges with the inactive fraction 16 from oranges only revealed no obvious differences between the samples (example shown in Figure 3A). After further separation of these fractions by a second HPLC gradient, activity was found primarily in fraction 14′ of both male- and female-derived samples (Figure 3B). By GC, the active fractions 14′

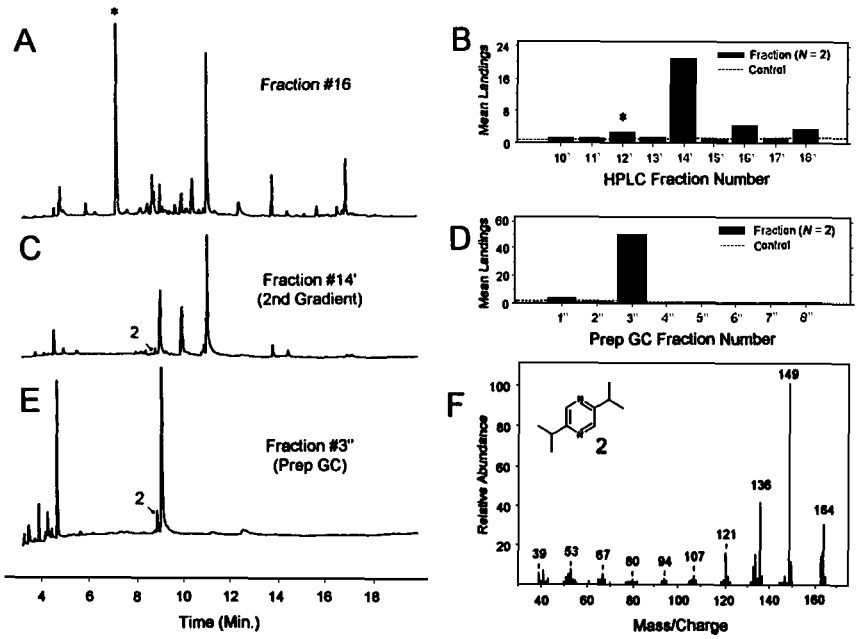

FIG. 3. (A) Gas chromatogram of active HPLC fraction 16. (B) Wind-tunnel bioassay results for fractions collected from the second HPLC gradient run of fraction 16 (fractions 1′–9′ contained only trace amounts by GC and had no bioassay activity). (C) Gas chromatogram of attractive fraction 14′ from the second HPLC gradient. Compound **2** was found in both male and female collections but was absent from unattractive orange collections. (D) Wind-tunnel bioassay results for preparative GC fractions collected from fraction 14′. (E) Gas chromatogram of active preparative GC fraction 3″. (F) Structure and mass spectrum of 2,5-diisopropylpyrazine (**2**). Asterisks in (A) and (B) indicate the presence of 2-phenylethanol (**3**) (see text).

had a small peak (compound **2**, retention time 9.3 min, Figure 3C) that was not detected in the inactive fraction 14′ from oranges.

Preparative GC and subsequent bioassays (Figure 3D and E) strengthened the association between compound **2** and activity. By GC, attractive fraction 3″ appeared to contain just two compounds, a larger peak and the smaller compound **2** peak (Figure 3E). The larger peak was also present in the corresponding fraction from the inactive, orange-derived sample. We conclude, therefore, that the compound **2** peak was responsible for the activity in fraction 3″. Only a small amount (<20 ng) of this compound was available for analysis.

GC-MS analysis of the compound **2** peak indicated a prominent molecular ion at m/z 164, a large M − 15 fragment (base peak), and a prominent M − 28

fragment (Figure 3F). A mass spectral library search found no compounds that matched the natural material. The overall appearance of the spectrum suggested an aromatic structure, possibly another phenol. A number of phenol analogs were examined by GC and MS to gain structural insight about the compound. However, it became apparent that a phenol analog could not be found to match the unknown with respect to both GC retention and mass spectral fragmentation. A high-resolution mass spectrum was obtained to determine whether heteroatoms other than oxygen were present. The observed mass of 164.1318 was in good agreement with the calculated mass for $C_{10}H_{14}N_2$ (164.1313). It was suspected that this compound might be a pyrazine, a common class of natural products containing two nitrogens. From a search of MS library spectra, isopropylmethylpyrazine had a fragmentation pattern similar to the unknown compound [a large M − 15 fragment (100%) and a prominent M − 28 fragment]. Although the molecular weight of isopropylmethylpyrazine (MW = 136) was too low to be the unknown, a diisopropylpyrazine (MW = 164) appeared to be a good possibility.

The rapid one-step synthesis of 2,5- and 2,6-diisopropylpyrazines provided a product that matched the natural material by both GC retention (9.33 min) and mass spectrum. The other synthetic product eluted slightly earlier (8.91 min), but had a very similar mass spectrum. It was unclear, however, whether the 2,5 or the 2,6 isomer corresponded to the natural product. The product of the second synthesis (only the 2,5 isomer) matched both the natural product and the later-eluting isomer from the first synthesis. Therefore, the natural product was 2,5-diisopropylpyrazine (**2**) (Figure 3F, structure).

Identification of 2-Phenylethanol. The largest peak, by GC analysis, in active HPLC fraction 16 (Figure 3A, asterisk) was identified as 2-phenylethanol (**3**) based on a mass spectral library search. Synthetic 2-phenylethanol matched the natural product in GC retention and mass spectrum. During the second HPLC gradient, 2-phenylethanol was collected in fraction 12′, which was slightly attractive in wind-tunnel bioassays (Figure 3B). 2-Phenylethanol, a very common natural product, had been previously reported as an attractant for *C. mutilatus* (Bartelt et al., 1993), and for this reason was studied further with *C. humeralis*.

Additional Unidentified Attractants. Preparative GC was used for isolating the active compound(s) in HPLC fraction 13. Wind-tunnel bioassays of the nine preparative GC fractions collected showed that only fraction 7′ from both beetle-derived samples was active (Figure 4B). The GC profile of fraction 7′ (Figure 4C) revealed five main peaks, three of which were present in roughly equal abundance in the corresponding unattractive fraction from oranges. The two largest peaks (unknown 1 at 13.09 min and unknown 2 at 13.24 min) were present in fractions derived from both sexes of beetles as well as the corresponding orange-derived fraction, but they were 10–20 times more abundant in the beetle-derived samples. The bioassay sample of fraction 7′ (Figure 4B) contained about 20 ng of unknown 1 and 100 ng of unknown 2.

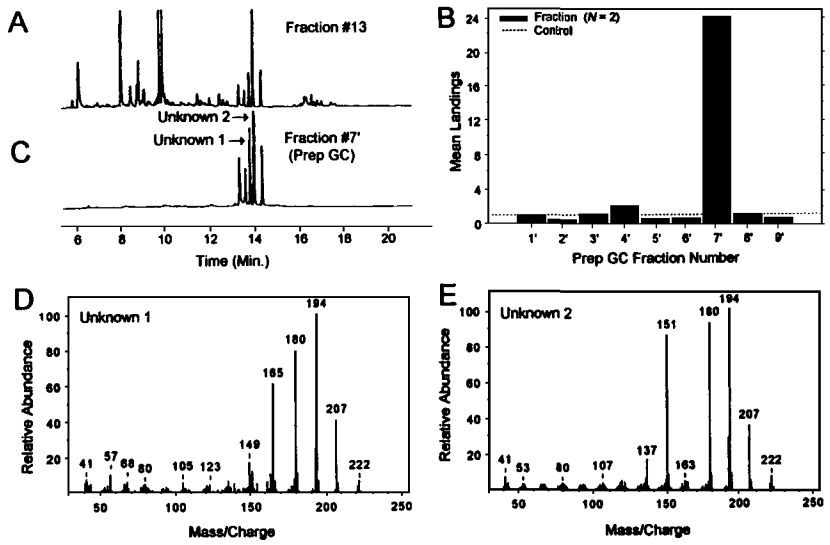

FIG. 4. (A) Gas chromatogram of active HPLC fraction 13. (B) Wind-tunnel bioassay results of preparative GC fractions collected from HPLC fraction 13. (C) Gas chromatogram of preparative GC fraction 7′ containing unknown compounds 1 and 2. (D and E) Mass spectra of unknown compounds 1 and 2.

Mass spectra of unknown 1 and unknown 2 (Figure 4D and E) were again suggestive of pyrazines. As with **2**, both spectra had large M – 15 and M – 28 fragments, but the molecular weights were higher (222 for both). A molecular weight of 222 suggested an oxygen was present (e.g., $C_{13}H_{20}N_2O$); pyrazines with free OHs or methoxy groups are frequently produced in nature by various microorganisms (Seitz, 1994). No matches were found for either compound in a library search of known spectra. However, certain characteristics in common with other structures existed. The large M – 57 (*m/z* 165) fragment in unknown 1 and the M – 71 (*m/z* 151) fragment in unknown 2 are characteristic of *sec*-butyl and isobutyl groups on pyrazines, respectively, by comparison with various library spectra. Unfortunately not enough material remained to do other analyses, and attempts to obtain these chemicals in larger amounts in subsequent collections were unsuccessful. Although they remain unidentified, either one or both of these compounds were probably responsible for the attractiveness of fraction 13 from the HPLC gradient.

*Dose Response Results for Attractants **1**, **2**, and **3**.* Bioassay results for tests of the three identified compounds at dose levels of 50, 5, and 0.5 ng, with and without PA, are shown in Figure 5. At the 50-ng level with PA, all three compounds were significantly more attractive than the control. Compound **2** (2,5-

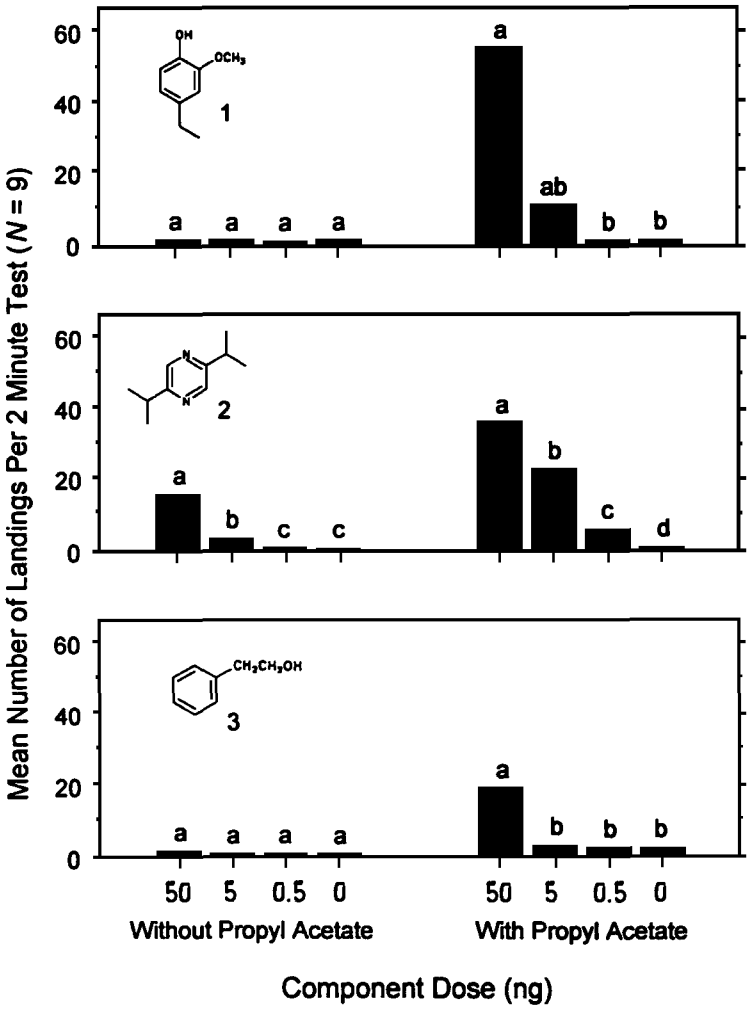

FIG. 5. Wind-tunnel bioassay results for 4-ethyl-2-methoxyphenol (**1**), 2,5-diisopropylpyrazine (**2**), and 2-phenylethanol (**3**), tested at four different dose levels, with and without propyl acetate coattractant. Within each group of four bars, treatments without letters in common differ significantly [HSD, 0.05, ANOVA in log (X + 1) scale].

diisopropylpyrazine), at 50 and 5 ng, was the only chemical that had significant activity when tested without the coattractant. With coattractant, compound **2** was also the only one that was active at the 5- and 0.5-ng levels.

Synergism Among Attractants 1, 2, and 3. Synergism among **1**, **2**, and **3**

(with PA) was dramatic when the compounds were tested at levels similar to those present in bioassays of the volatile collections (Figure 6). Of the single compounds, only **3** + PA was not significantly more active than PA alone (probably due to testing below its threshold level). As compounds were combined, a synergistic effect was seen. Of the two-way combinations, **1** + **2** was the most attractive, but any combination of two compounds was significantly more attractive than any single compound. The combination of all three chemicals was significantly more active than any two-compound combination. The combination of **1**, **2**, and **3** elicited two to four times as many landings as the two-way combinations and at least 10 times as many as any single compound.

Activity of Analogs of 4-Ethyl-2-methoxyphenol and 2,5-Diisopropylpyrazine. In addition to attractants **1**, **2**, and **3**, a number of other compounds were also identified from volatile collections, including other phenols and closely related methylethers. Examples are 4-allyl-2-methoxyphenol (**6**) and 4-ethylphenol (**8**) (both confirmed by GC and MS comparisons with authentic standards). It seemed possible that other phenol analogs besides 4-ethyl-2-methoxyphenol could also be attractive to *C. humeralis*. Similarly, given the suggestion of other active pyrazines (Figure 4), it seemed possible that pyrazines might have some general attractiveness.

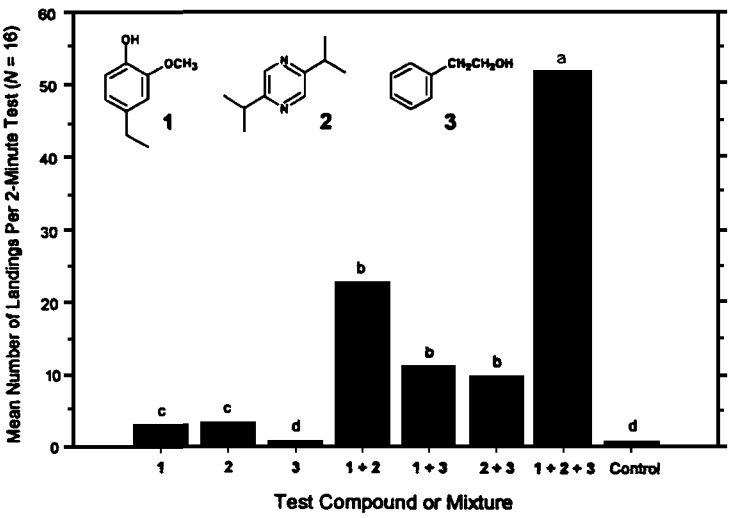

FIG. 6. Wind-tunnel bioassay results for 4-ethyl-2-methoxyphenol (**1**), 2,5-diisopropylpyrazine (**2**), and 2-phenylethanol (**3**), tested singly and in mixtures, at levels of 15, 2, and 35 ng, respectively. Propyl acetate was used as a coattractant with each test compound or mixture and also served as the control. Treatments without letters in common differ significantly [HSD, 0.05, ANOVA in log (X + 1) scale].

The results of tests with phenol and pyrazine analogs are presented in Figure 7, expressed as indices of activity (see Methods and Materials section). Only 2-methoxyphenol (**4**) had activity equivalent to 4-ethyl-2-methoxyphenol, and both compounds were the only ones to differ significantly from the control.

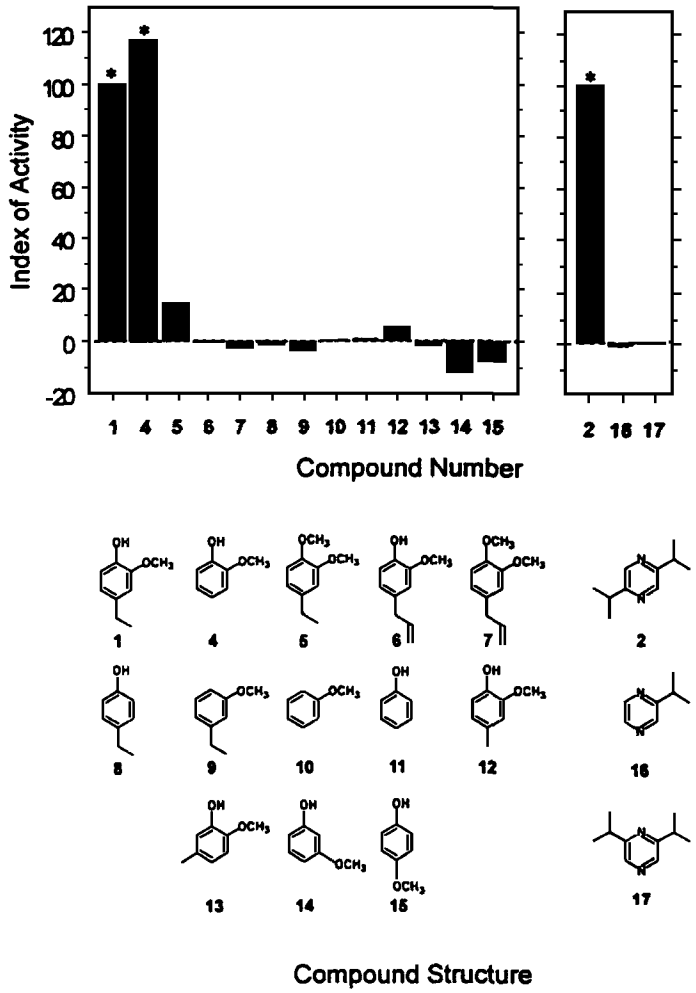

FIG. 7. Wind-tunnel bioassay results and compound structures for phenol analogs (**1**, **4–15**; 25 ng each) and pyrazine analogs (**2**, **16**, **17**; 2 ng each). Bioassay results are expressed as index of activity (see text). For test of **4–15**, standard compound was **1**; for tests of **16** and **17**, standard compound was **2**. Treatments with asterisks differed significantly from the propyl acetate control [HSD, 0.05, ANOVA in log (X + 1) scale].

For the pyrazines, neither **16** nor **17** elicited any detectable attraction, and only 2,5-diisopropylpyrazine differed significantly from the control.

Field Tests Using Compounds 1, 2, and 3, Food, and Carpophilus *Pheromones.* Field tests in South Carolina, Australia, and Israel produced very similar trends (Figure 8). In each test, the combination of pheromone, compounds **1**, **2**, and **3**, and food was the most attractive. Additionally, **1**, **2**, and **3**

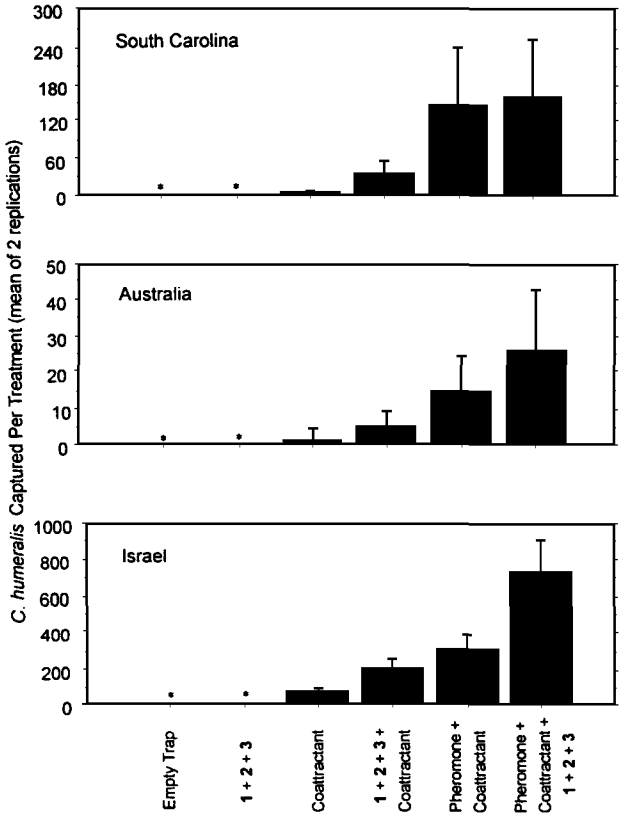

FIG. 8. Trap catches of *C. humeralis* from field tests in South Carolina, Australia, and Israel. **1 + 2 + 3** = 4-ethyl-2-methoxyphenol, 2,5-diisopropylpyrazine, and 2-phenylethanol on septum (500 μg each). Coattractant = bread dough (South Carolina and Australia) or grapefruit (Israel). Pheromone = 500 μg of *C. freemani* pheromone on septum (South Carolina) or 500 μg of *C. hemipterus* pheromone on septum (Australia and Israel). Treatment means and error bars (SE) were from ANOVA in log (X + 1) scale, after conversion back to numerical scale. Treatments with asterisks caught only four beetles total and were omitted from statistical analysis. In South Carolina, Australia, and Israel, lengths of trapping periods were eight, four, and four weeks, respectively.

with food attractant always captured more *C. humeralis* beetles than traps containing food attractant alone. Traps left empty or baited with just **1**, **2**, and **3** caught only four beetles (at the Israel site) and were omitted from statistical analysis.

The contrast for presence versus absence of compounds **1**, **2**, and **3** in the baits was significant ($P < 0.001$), while the interaction between the effect of **1**, **2**, and **3** and site was not ($P = 0.65$) (Table 1). This indicated that adding **1**, **2**, and **3** to baits caused a real increase in trap catches (overall, by a factor of 2.5), and the effect was consistent from site to site. The contrast for the presence versus absence of pheromone was significant ($P < 0.001$), and the interaction between pheromone effect and site ($P = 0.06$) was almost significant, which may have been due to two different pheromones being used. The interaction involving compounds **1**, **2**, and **3** and pheromone was also significant ($P = 0.03$) but was very small relative to the main effects.

*Source of Attractants **1**, **2**, and **3**.* The difference in attractiveness of volatiles between flasks containing beetles on oranges and those from oranges only was dramatic, but the source of the attractants was not initially clear. Possibilities were that: (1) they were released from oranges by beetle feeding, (2) they were being produced by both sexes of *C. humeralis*, or (3) they were produced by microorganisms growing on the fruit. To test the first possibility, volatile collections were repeated using pineapples, as it seemed unlikely that both types of fruit would contain the same three compounds.

TABLE 1. ANALYSIS OF VARIANCE OF FOUR TREATMENTS FROM COMBINED FIELD TEST RESULTS[a]

Source	DF	SS	MS	*F*	*P*
Site	2	8.022	4.011		
Blocks within sites	3	1.776	0.592		
Treatment	3	5.598	1.866	46.29	<0.001
1,2,3: absence vs presence	1	1.082	1.082	27.05	<0.001
Pheromone: absence vs presence	1	4.266	4.266	106.65	<0.001
1,2,3 × pheromone interaction	1	0.250	0.250	6.25	0.035
Treatment × site interaction	6	0.580	0.097	2.40	0.115
1,2,3 × site interaction	2	0.036	0.018	0.45	0.651
Pheromone × site interaction	2	0.319	0.160	4.00	0.057
1,2,3 × pheromone × site interaction	2	0.225	0.113	2.82	0.112
Residual	9	0.363	0.040		
Total	23	16.34			

[a]Treatments 1 and 2 omitted due to lack of response. Generall contrasts are shown for both treatment results and treatment × site interaction.

Flasks using pineapple as the host medium were always very attractive whether insects were present or not (results not shown). As with beetles on oranges, flasks containing beetles on pineapples become moldy and decomposed markedly within a few days. However, unlike orange control flasks, pineapple control flasks also become moldy quickly.

Production of **1**, **2**, and **3** was detected from flasks containing pineapple, suggesting the source was not oranges (Table 2). In addition, compounds **1**, **2**, and **3** were detected even from the pineapple controls, indicating the source was not the beetles. It appeared that some type(s) of microorganism was responsible for the production of **1**, **2**, and **3**.

To test this idea further, flasks of autoclaved pineapple pieces were inoculated with a small amount of pineapple material removed from a flask that was attractive and known to be producing **1**, **2**, and **3** (by GC). The inoculated fruit became visibly moldy after a few days and volatiles collected from these flasks were highly attractive in bioassays compared to volatiles from uninoculated flasks (which showed no indications of mold). Mean landings (N = 5) for inoculated and uninoculated flasks were 166 (± 45.6 SE) and 6 (± 0.5 SE), respectively. Average amounts, by GC analysis, of compounds **1**, **2**, and **3** produced in the inoculated pineapple flasks were 1800, 40, and 350 ng/day, respectively. No trace of **1**, **2**, and **3** could be found in the volatiles from the sterile pineapples. The fact that sterilized pineapple could be "seeded" with material from an active flask and begin producing the same attractive compounds within a few days, demonstrated that compounds **1**, **2**, and **3** were produced by microorganisms. (The observed production of **1**, **2**, and **3** in unsterilized control pineap-

TABLE 2. AMOUNTS OF COMPOUNDS **1**, **2**, AND **3** PRODUCED IN VOLATILE COLLECTORS CONTAINING PINEAPPLE

	Amount detected (ng/day)[a]					
	Compound **1**		Compound **2**		Compound **3**	
Volatiles from	Range	Mean ± SE[b]	Range	Mean ± SE	Range	Mean ± SE
♂ on Pineapple	100–1000	480 ± 165	ND[c]–100	70 ± 17	60–700	400 ± 127
♀ on Pineapple	40–500	208 ± 85	ND[d]–70	55 ± 15	50–1900	458 ± 361
Pineapple Control	50–1100	470 ± 180	ND[e]–80	80	500–3500	1380 ± 561

[a]Amoung recovered from volatile collectors/number of days collected.
[b]N = 5.
[c]ND = not detected in two replicates.
[d]ND = not detected in three replicates.
[e]ND = not detected in four replicates.

ple flasks was probably due to natural microflora or accidental contamination through handling). Measured amounts of compounds **1**, **2**, and **3** in 33 volatile collections from the inoculated flasks showed a strong correlation between **1** and **3** ($r = 0.96$, $P < 0.001$), but little correlation between **1** and **2** ($r = 0.23$, $P = 0.19$) or **2** and **3** ($r = 0.26$, $P = 0.14$). These data suggested that at least two different kinds of microorganisms were responsible for the production of compounds **1**, **2**, and **3**.

DISCUSSION

Natural Occurrence of 1, 2, and 3, and Related Compounds. 4-Ethyl-2-methoxyphenol (**1**) and 2-phenylethanol (**3**) are common natural products produced by various yeasts (Osaki et al., 1985). However, 2,5-diisopropylpyrazine (**2**) is reported here for the first time as a natural product, although other pyrazines are commonly produced by many microorganisms (Seitz, 1994).

Production of Attractants 1, 2, and 3. The production of **1**, **2**, and **3** in pineapple control flasks showed that these compounds were not *C. humeralis* pheromones, nor were they specific to oranges. Their occurrence in flasks containing sterile pineapple pieces inoculated with fragments of infected pineapple, demonstrated that microorganisms (as yet unidentified) were responsible for producing **1**, **2**, and **3**. It appears that orange pieces in controls were less susceptible to casual contamination than control pineapple pieces and that the establishment and continuance of microbial growth was more dependent on insect feeding in oranges than it was in pineapples.

Relationship of 1, 2, and 3 to Small Fermentation Volatiles. Previous studies on host and fungal volatiles attractive to *Carpophilus* species have focused on compounds typically associated with ripe or fermenting plant material. Smilanick et al. (1978) tested 19 compounds found in figs for *C. hemipterus* attraction and reported that a mixture of acetaldehyde, ethanol, and ethyl acetate was equal to the activity of fig paste. Phelan and Lin (1991) found they could achieve the full behavioral response of *C. hemipterus* to inoculated-banana odor by using a mixture of ethyl acetate, acetaldehyde, 2-pentanol, and 3-methyl-1-butanol. Within this mixture, substitution of compounds with other compounds or mixtures was possible while still maintaining full attraction. Lin and Phelan (1991) determined that a blend of acetaldehyde, 2-methyl-1-propanol, 3-methyl-1-butanol, and ethyl acetate or ethanol was comparable in attraction to fermenting bread dough for *C. lugubris* Murray.

Many of these same fermentation-associated compounds have been reported as attractants for *C. humeralis*. Yeast volatiles identified as attractants for the pineapple beetle are: ethanol, acetaldehyde, 2-methyl-1-propanol, 1-propanol, ethyl acetate, 3-methyl-1-butanol, and 2-methyl-1-butanol (Nout and Bartelt,

1998). Bartelt and Zilkowski (1998) matched the attractiveness of fermenting bread dough using a blend of these same compounds. They also demonstrated the attractiveness of a blend of acetaldehyde, ethanol, and ethyl acetate to *C. humeralis*. Smilanick et al. (1978) found acetaldehyde, ethyl acetate, and ethanol to be universally present in host fruits (including citrus and pineapple) of *Carpophilus* species. There is little doubt that most of these same compounds were present in the headspace volatiles of both oranges and pineapples in this study, but were not retained by the Super-Q filters.

While *C. humeralis* can use commonly occurring fermentation volatiles for food finding, the identification of **1**, **2**, and **3** demonstrates its ability to respond to a much greater range of food chemicals than might have been expected. Phelan and Lin (1991) suggested that host-finding by polyphagous insects could be mediated by a response to ubiquitous plant volatiles or by a response to any number of more specific components. For *C. humeralis*, a combination of both groups appears to produce the most effective attractants.

Phenol and Pyrazine Structure–Activity Relationships. C. humeralis was attracted to only one of the 14 analogs of **1** and **2** that were tested, signifying that the species has rather specific structural requirements for certain phenols and pyrazines. No attraction was detected to either 2-isopropylpyrazine or 2,6-diisopropylpyrazine, the closely related analogs of compound **2**. Among the phenol analogs, only 2-methoxyphenol (**4**) was significantly active, indicating that a methoxy group in the 2 position was critical for attraction and the 4 position required either an ethyl group or no group at all. Phenols were always more attractive than the corresponding methyl ethers. Curiously, 2-methoxy-4-methylphenol (**12**), the compound structurally intermediate between **1** and **4** (having a methyl group in the 4 position instead of an ethyl group or hydrogen), was not attractive.

In a similar study, McGovern and Ladd (1988) examined a series of 2-methoxyphenols, 1,2-dimethoxyphenols, and 1,3-benzodioxoles for the structural features important for attraction to *Diabrotica barberi* Smith & Lawrence (Coleoptera: Chrysomelidae). They reported that a methyl, ethyl, propyl, or butyl alkyl chain at the 4 position of 2-methoxyphenols was necessary for attraction, with a 3-carbon chain being optimal. In addition, attraction was lost when a methoxy group was substituted for the hydroxyl group in the first position. Preference in *C. humeralis* for specific phenolic compounds appears more limited than that of *D. barberi*.

Synergism Among Compounds 1, 2, and 3. Attraction by *C. humeralis* to each of compounds **1**, **2**, and **3**, individually, was limited. However, the synergistic activity of **1**, **2**, and **3** combined, resulted in dramatic *C. humeralis* attraction in wind-tunnel bioassays, especially when further synergized with propyl acetate. Increased attraction due to synergism of compounds is not unusual for *Carpophilus* species; Smilanick et al. (1978) reported that the combination

of acetaldehyde, ethyl acetate, and ethanol was significantly more active for *C. hemipterus* than any 2-compound combination. The attractiveness of *Carpophilus* pheromones is synergized dramatically by food volatiles such as fermenting bread dough odor (Bartelt, 1997).

For *C. humeralis*, attraction towards compounds **1** and **2** may be synergized by many food-related compounds in addition to 2-phenylethanol. Compound **3** gave little indication of activity during the initial examination of the volatile collections, and it was investigated further only because of its previously reported attraction to *C. mutilatus* and its relative abundance in fraction 16. It is possible that many other unstudied compounds within the volatile collections could have synergized **1** and **2** just as effectively. Additionally, lower-weight compounds not retained by the Super-Q filter may also synergize compounds **1** and **2**. Many alcohols, such as methanol, ethanol, and 1-propanol, as well as a number of acids and esters, are frequently found in fermenting foods and are synergists for the pheromone of *C. hemipterus* (Dowd and Bartelt, 1991). The possibility exists that many of these compounds could lead to similar or even greater synergism of **1** and **2** over that which was observed with compound **3**.

C. humeralis *Field Trials.* The field trials showed that adding a mix of compounds **1**, **2**, and **3** to other *C. humeralis* attractants dramatically increased trap catches for this species. There is potential to increase trap catches further by adjusting the doses of each of the components involved.

For the pineapple beetle, the attractiveness of **1**, **2**, and **3** could play an important role in developing traps for the control of this species. However, the identification of these microbe-produced compounds suggests that host finding for *C. humeralis* is complex and that further studies may find many other attractive compounds that are as good or even better than those reported here.

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COMPARATIVE DETOXIFICATION OF PLANT (*Magnolia virginiana*) ALLELOCHEMICALS BY GENERALIST AND SPECIALIST SATURNIID SILKMOTHS

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Abstract—The foliage of sweetbay magnolia (*Magnolia virginana*) contains at least two biologically active phenylpropanoid compounds (magnolol and a biphenyl ether) that are toxic to a number of generalist insect herbivores. These compounds have little effect on caterpillars of the sweetbay silkmoth, *C. securifera*, which is a specialist on sweetbay, but they are toxic to two closely related silkmoths, *C. angulifera* and *C. promethea*. To understand the influence of phytochemistry on the evolution of host use and feeding specialization in *Callosamia*, the detoxification capability of *C. securifera* was compared with that of *C. angulifera* and *C. promethea*. Degradation of magnolol and the biphenyl ether by midgut homogenate of the sweetbay specialist was NADPH-dependent and inhibited by piperonyl butoxide, suggesting the involvement of cytochrome P-450 detoxification enzymes. Both were degraded three times faster in the specialist compared to the unadapted herbivores. Higher rates of degradation could not be induced in the polyphagous *C. promethea* by a mixture of magnolol and the biphenyl ether or by the P-450 inducer pentamethylbenzene, nor did activity vary significantly when larvae were reared on different host plants. Use of sweetbay by *Callosamia* silkmoths appears to be dependent on their ability to degrade host toxins rapidly via midgut detoxification enzymes. Moreover, the intraspecific comparisons contradict the common prediction that higher levels of cytochrome P-450 activity are found in more polyphagous species; instead, P-450 activity is more closely associated with specific chemical attributes of the herbivores' host plants.

Key Words—Insect detoxification enzymes, phenylpropanoids, Saturniidae, *Magnolia virginiana*, *Callosamia*, feeding specialization.

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INTRODUCTION

The ability of herbivores to tolerate or detoxify ingested plant allelochemicals is recognized as an important determinant of both ecological and evolutionary patterns of host use in many taxa. Specific physiological or biochemical characteristics of herbivores may constrain them to a subset of plants in the environment or allow radiation and colonization of novel hosts (Ehrlich and Raven, 1964). Not only does the capacity for biochemical detoxification of host allelochemicals likely play a role in the evolution of specific host utilization patterns within herbivore lineages, but the metabolic costs associated with such detoxification mechanisms have been proposed to be the basis of physiological trade-offs between the evolution of specialist versus generalist feeding habits (Whittaker and Feeny, 1971; Krieger et al. 1971; Futuyma and Moreno, 1988). According to the metabolic load hypothesis, the maintenance of detoxification enzymes is metabolically costly, so insects that specialize on one or a few hosts gain a metabolic advantage over generalists in that they encounter fewer allelochemicals and hence can allocate fewer resources to detoxification systems.

Detoxification of plant compounds in insects occurs via the action of oxygenases, reductases, hydrolases, and transferases that are located primarily in the fat body and midgut tissues and presumably evolved for that purpose (Brattsten, 1979; Dowd et al., 1983; Ahmad et al., 1986; Lindroth, 1989, 1991). Of these systems, the microsomal cytochrome P-450 based mixed-function monooxygenases (MFOs) or polysubstrate monooxygenases (PSMOs) are the most important general-purpose detoxification mechanism for metabolizing host allelochemicals (Ahmad, 1979; Brattsten, 1979). Their broad substrate specificity and the inducible nature of the PSMO system are consistent with their proposed central role in polyphagous feeding habits, since generalist herbivores can be expected to encounter a broad array of secondary metabolites in their diet (Krieger et al., 1971; Ahmad, 1983). Early comparisons of PSMO activity in generalist and specialist insects supported this hypothesis and led to the generalization that higher PSMO activity is correlated with broad diet breadth in herbivorous insects (Krieger et al., 1971; Ahmad, 1983; Yu, 1987).

In a comparison of an array of unrelated insect herbivores, however, Rose (1985) found no correlation between the degree of polyphagy and PMSO activity, as measured by aldrin epoxidation. Subsequent studies on a variety of insects have shown that PSMO and other detoxification enzyme activities are not always correlated with broad host range. Examples of equal or higher activity in specialist herbivores have been demonstrated for PSMO-based detoxification activity (Berenbaum, 1991; Bull et al., 1984, 1986; Neal, 1987; Nitao, 1989; Rose, 1985), glutathione transferases (Lee and Berenbaum, 1992; Gunderson et al., 1986; Wadleigh and Yu, 1988), esterases (Lindroth, 1989, 1991), and various antioxidant enzymes (Aucoin et al., 1991; Berenbaum, 1991; Lee and Berenbaum, 1992;

Pardini et al., 1989). These findings suggest that differences between detoxification activities of specialists and generalists may be better correlated with specific chemical characteristics of the host plant and perhaps involve differences in inducibility and substrate specificity rather than constitutive levels of activity (Berenbaum, 1991; Nitao, 1995).

The wild silkmoth genus *Callosamia* (Saturniidae) serves as a good model system to examine simultaneously the relationship between diet breadth, host chemistry, and patterns of detoxification activity within a phylogenetic context. The genus is composed of three closely related species that display various degrees of host specialization mediated in part by host phytochemistry. *C. promethea* is highly polyphagous, feeding on trees from over five different plant families, including black cherry (*Prunus serotina* Ehrhart, Rosaceae), ash (*Fraxinus* sp., Oleaceae), sassafras [*Sassafras albidum* (Nuttall) Nees, Lauraceae], spicebush [*Lindera benzoin* (*L.*), Lauraceae], tuliptree (*Liriodendron tulipifera* L., Magnoliaceae), sweetgum (*Liquidambar styraciflua* L., Hamamelidaceae), lilac (*Syringa vulgaris* L., Oleaceae), and others. *C. angulifera* is a semispecialist on a subset of hosts used by *C. promethea*, most commonly feeding on tuliptree. The third species, *C. securifera*, is a strict specialist on another magnoliaceous host, sweetbay magnolia (*Magnolia virginiana* L., Magnoliaceae). Interestingly, neither *C. promethea* nor *C. angulifera* feed on sweetbay magnolia in the wild (with few exceptions), and neither is able to survive on it in laboratory assays. Sweetbay foliage contains two biologically active neolignan compounds, magnolol and a biphenyl ether, that are toxic to insects (Nitao et al., 1991, 1992). When painted on leaves of an acceptable host (tuliptree) at concentrations equivalent to those found in sweetbay, both compounds reduce the growth and survival of early instar *C. promethea* and *C. angulifera*, but have no effect on *C. securifera* (Johnson et al., 1996a). Thus, the use of sweetbay by members of this genus appears to require physiological and/or biochemical adaptations for coping with these allelochemicals.

The purpose of this study was to evaluate the adaptive significance of enzymatic detoxification systems in the evolution of host use patterns in silkmoths by characterizing and comparing the enzymatic detoxification of magnolol and the biphenyl ether among *Callosamia* species and by testing whether detoxification activity is better correlated with the degree of polyphagy or the use of a specific host plant (sweetbay).

METHODS AND MATERIALS

Insects. Larvae used in these experiments were first or second generation offspring from wild-collected adults and cocoons. *C. securifera* larvae originated from livestock collected in Lake and Highlands counties, Florida. *C. promethea*

were collected from wild populations in southern Wisconsin and Michigan, and *C. angulifera* in southern Michigan. Larvae were reared on foliage harvested locally from trees in Ingham county, Michigan, or from potted sweetbay trees maintained at Michigan State University for several years (original source: Herren Nursery, Florida Division of Forestry). Detached leaves were kept fresh by placing the petioles in water-filled plastic vials with rubber caps and were replaced every other day. Caterpillars were reared in groups of 5–10 in plastic shoeboxes at 24°C on an 18L : 6D photoperiod.

Purification of Allelochemicals from Sweetbay. Magnolol and the biphenyl ether used in the in vitro metabolism experiments were purified from sweetbay magnolia leaves as described in Johnson et al. (1996a). Briefly, freeze-dried leaves were milled to a powder and sequentially extracted with hexane, ethyl acetate, and methanol. Magnolol (5,5′-diallyl-2,2′-dihydroxybiphenyl), the biphenyl ether (4,4′-diallyl-2,3′-dihydroxybiphenyl ether) and methoxyhonokiol (3,5′-diallyl-2′-hydroxy-4-methoxybiphenyl) were isolated from the hexane extract using centrifugal partition chromatography, then further purified by flash column (Sigma silica 60 Å eluted with hexane–ether 4 : 1) and preparatory thin layer chromatography (silica gel, 2000 μm tapered Uniplate, Analtech, Newark, Delaware; developed twice in hexane–ether 4 : 1). For in vitro metabolism experiments, magnolol and the biphenyl ether were presented as a 2 : 3 mixture to approximate the natural proportions found in sweetbay foliage (Nitao et al., 1991). Methoxyhonokiol was used only as an internal standard during HPLC quantification.

Characterization of Detoxification Enzymes of Caterpillar Midgut Tissues. Preliminary experiments with midgut homogenate showed that magnolol and the biphenyl ether were rapidly degraded by fresh, but not boiled tissue, indicating the presence of an enzymatic detoxification mechanism. Two typical characteristics of cytochrome P-450 detoxification systems are that they are dependent on NADPH and sensitive to the specific P-450 inhibitor, piperonyl butoxide (Brattsten, 1979).

The NADPH dependency of magnolol and the biphenyl ether metabolism by *C. securifera*, *C. promethea*, and *C. angulifera* midgut tissue was measured as follows: *C. securifera* larvae were reared on sweetbay, and *C. promethea* and *C. angulifera* on tuliptree. Midguts from 2- to 4-day-old third instar larvae were removed, sliced longitudinally, and rinsed with chilled 0.1 M potassium phosphate buffer (pH 7.8; 0.05 M EDTA). Groups of 5–10 midguts were homogenized in a hand-held Ten Broeck homogenizer in phosphate buffer (1 ml/g larval wet mass) and the crude homogenate was used immediately.

Detoxification of magnolol and the biphenyl ether was estimated by the rate of disappearance of the parent compared during in vitro incubation with midgut homogenate. Reactions were begun with the addition of a mixture of 10 μg of magnolol and 15 μg biphenyl ether in 20 μl methanol to an incubation

mixture of 0.1 M potassium phosphate buffer (pH 7.8) and 1.0–3.0 mg midgut protein with or without 1.0 mM NADPH in uncapped glass scintillation vials for a total volume of 1.0 ml. After 30 min at 30°C in a shaking water bath, reactions were stopped by adding 250 μl 1.0 N hydrochloric acid, and the vials were immediately placed on ice. Inactivated (boiled) homogenate was included as a control. Each incubation was done in duplicate and the experiment was replicated two to six times for each species.

The magnolol and biphenyl ether remaining in each vial after the incubation was extracted with organic solvent and quantified by high-pressure liquid chromatography. After addition of an internal standard (10 μg of methoxyhonokiol), samples were extracted with two volumes of ethyl acetate, dried under an airstream, then redissolved in 500 μl methanol. Samples were filtered through a nylon filter (Alltech 0.45 U) and then injected into a Beckman Series model 167 high-performance liquid chromatograph with ultraviolet detector. Magnolol, biphenyl ether, and methoxyhonokiol were separated on a C-18 column (150 mm $\times$ 4.6 mm OD) eluted with 80% methanol at a flow rate of 1.5 ml/min and quantified by comparison with standard curves. Unrecovered compound was assumed to have been metabolized. Significant differences in the amount of magnolol and biphenyl ether degraded by active tissue with and without NADPH were determined by t test at $P < 0.05$. The amount of tissue in each incubation was estimated from an aliquot of homogenate using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, California) with bovine serum albumin as a standard.

The effect of piperonyl butoxide (PBO), a methylenedioxyphenyl cytochrome P-450 inhibitor, on the degradation of magnolol and biphenyl ether was assessed using similar assay conditions. Because *C. angulifera* caterpillars were unavailable at the time of the experiment, the effects of PBO were tested only on *C. promethea* and *C. securifera* larvae. Midgut homogenate was prepared from mid-fourth-instar larvae (five to seven per replicate) as described previously. Homogenate (100–300 μl) was incubated with a mixture of 10 μg magnolol and 15 μg biphenyl ether and several concentrations (0, 10^{-2}, 10^{-3}, and 10^{-4} M) of PBO (Aldrich Chemical Company, Milwaukee, Wisconsin; technical grade 90%). PBO was added in 10 μl methanol to a total incubation volume of 1.0 ml potassium phosphate buffer (pH 7.8) supplemented with 0.3 mM NADPH. Checks consisted of 10^{-2} M piperonyl butoxide, NADPH, substrate, and tissue inactivated by the addition of 250 μl hydrochloric acid at the beginning of the reaction. The mixtures were incubated for 10 minutes at 30°C, then stopped by the addition of 250 μl 1.0 N hydrochloric acid and immediate chilling. Undegraded magnolol and biphenyl ether in the incubation vials were extracted and quantified as described previously. Dose-dependent effects of piperonyl butoxide on magnolol and biphenyl ether degradation were analyzed separately using a polynomial regression ANOVA model (SAS Institute, 1985).

Induction of Detoxification Enzymes by Sweetbay Allelochemicals and Pen-

tamethylbenzene. Two experiments were conducted to determine whether detoxification of magnolol and the biphenyl ether in the polyphagous *C. promethea* could be induced to higher levels. In the first experiment, 1 to 3-day-old third-instar larvae were fed sassafras leaves painted with a 2:3 mixture of magnolol–biphenyl ether in methanol (0.2:0.3% w/w fresh leaf), 0.2% pentamethylbenzene in methanol (Aldrich), or methanol for 24 hr, then switched to untreated foliage for 24 hr prior in vitro assays. Pentamethylbenzene is a synthetic cytochrome P-450 inducer that increases detoxification enzymes in many insects. Larvae were housed individually in plastic Petri dishes lined with moistened filter paper and leaves were kept fresh in water-filled plastic vials. After dissection, larval midguts were pooled ($N = 4$) and homogenized. The experiment was performed twice. Degradation of magnolol and the biphenyl ether was assayed as described earlier.

The second experiment differed in that the inducing agents were applied to tuliptree, rather than sassafras, and larvae were allowed to feed for a total of 48 hr before being switched to untreated leaves. Because of the differences in protocol, the two experiments were analyzed separately for differences in rates of in vitro degradation of magnolol and the biphenyl ether using one-way ANOVA (Systat) (Wilkinson, 1990).

Comparative Detoxification of Sweetbay Neolignans by C. securifera, C. promethea *and* C. angulifera. The rates of in vitro degradation of magnolol and the biphenyl ether by third-instar *C. securifera* caterpillars were compared to those of similar stage *C. angulifera* and *C. promethea*. *C. securifera* larvae were reared on sweetbay (its only host), *C. angulifera* on tuliptree (its preferred host), and three groups of *C. promethea* were reared on tuliptree, sassafras, and black cherry, respectively. The larval host plant could not be standardized because *C. securifera* survives poorly on tuliptree, and neither *C. promethea* nor *C. angulifera* can be reared on sweetbay. Fresh midgut homogenates were prepared (three to five pooled guts) and assays performed in duplicate as described earlier, with two to six replicate trials conducted on different days for each species–host–plant combination. A mean rate of degradation for each species–host combination was calculated from the replicate trials and differences between means tested using a Tukey-Kramer test after one-way ANOVA confirmed significant differences (Systat) (Wilkinson, 1990).

RESULTS

Characterization of Detoxification of Sweetbay Neolignans in Callosamia. Addition of NADPH to incubation mixtures significantly enhanced degradation of the biphenyl ether in *C. securifera* and *C. promethea* (Table 1). Its effects on biphenyl ether degradation by *C. angulifera* tissue was not statistically analyzed

TABLE 1. IN VITRO DEGRADATION OF BIPHENYL ETHER AND MAGNOLOL BY MIDGUT HOMOGENATE OF THIRD-INSTAR *C. securifera*, *C. angulifera*, and *C. promethea* LARVAE IS NADPH-DEPENDENT[a]

Treatment	*N*	Amount degraded (nmol/vial)	
		Biphenyl ether	Magnolol
C. securifera			
NADPH	3	48.1 ± 0.0[b]	31.2 ± 1.9[b]
No NADPH	3	18.6 ± 2.2	4.9 ± 2.6
NADPH stopped	3	16.4 ± 4.5	6.0 ± 3.0
No NADPH stopped	3	8.3 ± 5.1	2.3 ± 1.5
C. promethea			
NADPH	6	24.0 ± 4.8[b]	16.9 ± 3.8
No NADPH	6	17.9 ± 2.9	16.2 ± 3.8
NADPH stopped	6	6.7 ± 1.3	10.2 ± 3.0
No NADPH stopped	6	9.0 ± 1.6	9.8 ± 0.3
C. angulifera			
NADPH	2	13.5 ± 0.3[c]	13.9 ± 0.08[c]
No NADPH	2	12.8 ± 0.3	10.2 ± 1.9
NADPH stopped	2	7.1 ± 2.3	6.4 ± 0.04
No NADPH stopped	2	5.8 ± 2.6	3.8 ± 1.1

[a]"Stopped" treatments consisted of midgut homogenate inactivated by the addition of 1 N HCl at the beginning of the incubation and served as checks. Values are means ± SE of multiple homogenization events (*N*) with duplicate incubations.

[b]Denotes significant difference between NADPH and no NADPH treatments determined by *t* test, $P < 0.05$.

[c]Not tested statistically because of small sample size and unequal variances.

because of unequal variances and small sample size. Biphenyl ether degradation was inhibited by PBO in a dose-dependent fashion (Table 2). At a concentration of 10^{-2} M, PBO reduced biphenyl ether metabolism by two fifths in *C. securifera* and to less than one tenth the activity of the control in *C. promethea* (Figure 1). In *C. securifera*, the degree of inhibition is likely an underestimation, since the uninhibited control appeared to have already become substrate-limited before the incubations were halted.

In contrast, magnolol degradation was NADPH dependent only in *C. securifera;* addition of NADPH did not significantly increase metabolism of this neolignan in *C. promethea* or *C. angulifera* (Table 1). Likewise, the P-450 inhibitor PBO reduced metabolism of the compound in *C. securifera* tissue, but had no significant effect on magnolol metabolism by *C. promethea* (Table 2).

Induction of Detoxification Enzymes by Sweetbay Allelochemicals and Pentamethylbenzene. Treatment of leaves with pentamethylbenzene or the neolignan mixture for 24 or 48 hr did not enhance rates of biphenyl ether or magnolol

TABLE 2. ANOVA OF DOSE-DEPENDENT INHIBITION OF BIPHENYL ETHER AND MAGNOLOL DEGRADATION BY PIPERONYL BUTOXIDE IN *C. securifera* AND *C. promethea*

Source	Biphenyl ether				Magnolol			
	SS	df	*F*	P	SS	df	*F*	P
C. securifera (specialist)								
Treatment	167.40	3	5.99	0.019	24.12	3	2.35	0.124
Linear	154.27	1	16.57	0.004	22.16	1	6.48	0.026
Quadratic	11.94	1	1.28	0.290	1.95	1	0.57	0.464
C. promethea (generalist)								
Treatment	177.62	3	8.84	0.031	15.77	3	4.41	0.093
Linear	162.37	1	24.24	0.008	0.66	1	0.00	0.450
Quadratic	10.46	1	1.56	0.280	15.07	1	12.65	0.024

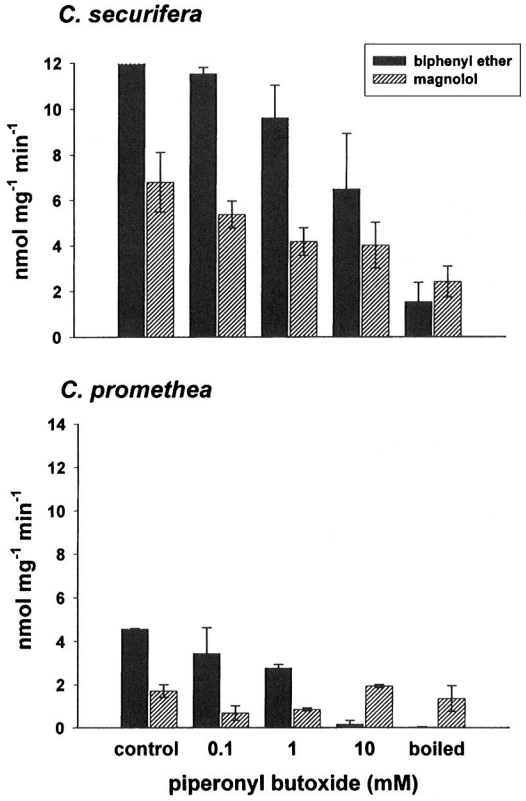

FIG. 1. Piperonyl butoxide inhibition of magnolol and biphenyl ether degradation by midgut homogenates of *C. securifera* and *C. promethea*. Data shown are means ±SE.

TABLE 3. DEGRADATION OF SWEETBAY ALLELOCHEMICALS BY *C. promethea* LARVAE[a]

Treatment	Degradation (nmol/min/mg)		N
	Biphenyl ether	Magnolol	
Caterpillars fed inducers painted on sassafras leaves for 24 hr			
Control	4.17 ± 0.51	3.79 ± 0.27	2
Pentamethylbenzene	3.38 ± 1.07	3.06 ± 0.35	2
Biphenyl ether–magnolol	5.50 ± 0.53	3.76 ± 0.41	2
Caterpillars fed inducers painted on tuliptree leaves for 48 hr			
Control	5.38 ± 1.11	4.55 ± 0.34	2
Pentamethylbenzene	5.29 ± 0.87	4.49 ± 0.34	2
Biphenyl ether–magnolol	5.20 ± 0.85	4.03 ± 0.64	2

[a]Larvae were exposed to pentamethylbenzene (a cytochrome P-450 inducer) or a mixture of the biphenyl ether and magnolol painted on sassafras leaves for 24 hr and tuliptree leaves for 48 hr. Values are the means ± SE of two homogenization events, each with five to seven pooled midguts and duplicate incubations.

degradation compared to *C. promethea* larvae fed leaves treated with methanol (Table 3).

Comparative Detoxification of Sweetbay Neolignans by C. securifera, C. promethea *and* C. angulifera. *C. securifera* midgut tissue degraded magnolol and the biphenyl ether two to three times more rapidly than tissue from the two species unadapted to sweetbay (Figure 2). The mean rate of magnolol degradation was 7.52 ± 0.94 nmol/min/mg for *C. securifera* compared to 2.89 ± 0.15 nmol/min/mg for *C. angulifera* and 2.82 ± 0.56 nmol/min/mg for *C. promethea* reared on tuliptree. Biphenyl ether degradation by *C. securifera* tissue was 13.62 ± 4.81 nmol/min/mg compared to 2.88 ± 0.71 nmol/min/mg and 4.81 ± 1.12 nmol/min/mg by *C. angulifera* and *C. promethea*, respectively. Detoxification activity in the highly polyphagous *C. promethea* was not significantly different from that of the oligophagous *C. angulifera*, nor did the host plant significantly alter the rate of metabolism of the allelochemicals. Rates of magnolol degradations were 2.82 ± 0.56, 2.26 ± 0.64, and 2.07 ± 0.45 nmol/min/mg (ANOVA $P = 0.47$) and biphenyl ether degradation 4.81 ± 1.12, 2.24 ± 1.12, 3.04 ± 0.80 nmol/min/mg ($P = 0.21$) on tuliptree, sassafras, and black cherry, respectively, in *C. promethea*.

DISCUSSION

Host-plant phytochemistry exerts a strong influence on patterns of larval survival and host use in *Callosamina* silkmoths, particularly on magnolia-

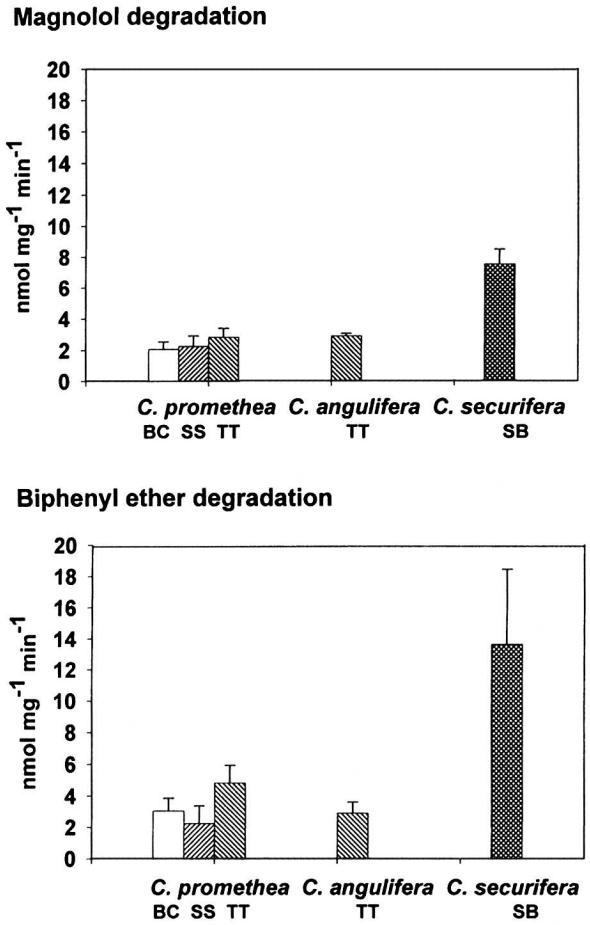

FIG 2. Comparative rates of in vitro degradation of sweetbay allelochemicals (magnolol and the biphenyl ether) by third-instar larvae of the three silkmoth species. The letters under the species names indicate the host plant on which larvae in the assays were reared (BC = black cherry, SS = sassafras, TT = tuliptree, and SB = sweetbay). Data shown are means ± SE.

ceous hosts. *C. securifera*'s ability to successfully feed and develop on sweetbay appears to be correlated with rapid enzymatic metabolism of two toxic phenylpropanoids, magnolol and a biphenyl ether, which present a phytochemical barrier to unadapted herbivore species (Johnson et al., 1996a). Not only are these compounds toxic to unadapted *Callosamia*, but also to unadapted Lauraceae-feeding swallowtail butterflies and the extremely polyphagous fall webworm,

Hyphantria cunea (Dur.)(Arctiidae) (Nitao et al., 1992). Although *C. promethea* and *C. angulifera* larvae also metabolize magnolol and the biphenyl ether, the rates of degradation are considerably slower than in *C. securifera*.

Although many details of absorption, transport, and metabolism of these compounds are still unknown, the preliminary characterization of biphenyl ether metabolism by midgut homogenates of *C. securifera* and *C. promethea* shows that it is NADPH-dependent and inhibited by piperonyl butoxide, traits that are characteristic of cytochrome P-450 enzymes. This is not surprising, since cytochrome P-450s are generally considered to be one of the most important detoxification systems in insects, particularly for ingested plant compounds (Brattsten, 1992). In addition, it has been shown that metabolism of biphenyl compounds in fall armyworms occurs by hydroxylation via PSMOs (Yu and Ing, 1984). In mammals, biphenyl is metabolized by microsomal cytochrome P-450s to a variety of hydroxybiphenyls, which are then conjugated with glucuronic acid and/or sulfate and excreted (Powis et al., 1991; Pacific, 1991).

Although degradation of ingested magnolol in *C. securifera* also appears to be via cytochrome P-450s, metabolism in the more polyphagous *C. promethea* occurred more slowly and was not sensitive to NADPH or piperonyl butoxide. These differences suggest that the cytochrome P-450 enzymes required for magnolol metabolism may be absent or at least that their activity is very low. Although cytochrome P-450s are collectively able to detoxify a wide variety of substances, specific P-450 forms are often necessary to degrade individual toxins. Such substrate specificity in P-450 isozymes has been shown to be the basis for insecticide resistance in houseflies (Feyereisen et al., 1989; Carino et al., 1992) and xanthotoxin metabolism in umbellifer specialists (Berenbaum et al., 1992; Cohen et al., 1989, 1992; Ma et al., 1994). In mammals, orally administered magnolol is extensively conjugated with glucuronides and other conjugates in the liver, and then isomerized or reduced, but the role of cytochrome P-450s in these processes is unclear (Ma et al., 1988; Tsai et al., 1996). In silkmoth caterpillars, it is possible that multiple detoxification mechanisms are at work, one or more with relatively low efficiency (present in both *C. promethea* and *C. securifera*) and another involving cytochrome P-450s (in *C. securifera*).

The rates of allelochemical degradation by gut homogenate in this study (magnolol ranged from 2.07 to 7.52 nmol/mg/min and the biphenyl ether from 2.24 to 13.62 nmol/mg/min) were within the range of in vitro rates typically reported for crude homogenate or microsomal preparations. Degradation of xanthotoxin by slices of midgut tissue in the black swallowtail (*P. polyxenes*) ranges from 0.9 to 1.6 nmol/mg/min (Bull et al., 1984); metabolism of the cardenolide uscharidin by monarch (*Danaus plexippus*) caterpillar gut homogenate ranges from 1.97 to 4.99 nmol/mg/min (Marty and Krieger, 1984). Salicin is degraded at much higher rates (304.2 ± 6.3 nmol/mg/min) by *Papilio glaucus* β-glucosidases (Lindroth, 1988). It should be noted that the estimates of detoxification

activity in our study may be somewhat inflated by incomplete extraction of the parent compound from the incubation mixture, since only the disappearance of the compound was quantified. Nonetheless, the average recovery of the internal standard (methoxyhonokiol) was modest (around 60%), but consistent among trials and species, so the observed differences among treatments are not likely affected by extraction efficiency.

The physiological mode of action of magnolol and the biphenyl ether in insects is not known, although they appear to act as toxins, not simple feeding deterrents (Johnson et al., 1996a). Caterpillars in captivity feed readily on sweetbay foliage or on other leaves painted with the compounds for several days before dying; they also do not selectively avoid leaf disks painted with compounds in choice tests (Johnson, unpublished data). Many lignans have biological activity (MacRae and Towers, 1984; Bernard et al., 1989; Faure et al., 1990) and both magnolol and the biphenyl ether have been shown to have a broad spectrum of antimicrobial and insecticidal activity (Clark et al., 1981; Nitao et al., 1991, 1992). Sarker (1997) recently reviewed the biological activity of magnolol. In mammals, it affects the central nervous system (Tsai et al., 1995), inhibits muscle contractions (Teng et al., 1990), exerts neurotrophic activity in cultured cells (Fukuyama et al., 1992), has antiinflammatory and analgesic effects (Wang et al., 1995), inhibits xanthine oxidase (Chang et al., 1994), and has antioxidant properties (Lo et al., 1994; Fugita and Taira, 1994; Hong et al., 1996; Haraguchi et al., 1997; Ogata et al,, 1997). Relatively little is known about the pharmacological properties of the biphenyl ether.

For simplicity, I have assumed that the higher detoxification activity observed in *C. securifera* is due to the inherent physiology of the animal, not dietary factors. However, because cytochrome P-450 and other detoxification enzymes are often induced by exposure to specific toxins, we cannot rule out the possibility that detoxification activity in *C. securifera* is induced by compounds present in sweetbay foliage, such that the higher activities measured are a consequence of the diet rather than (or in addition to) the inherent physiology of the animal. This is difficult to test experimentally because neither *C. promethea* nor *C. angulifera* survive on sweetbay long enough to quantify their detoxification activities while feeding on this host. However, attempts to induce higher detoxification activity in *C. promethea* by pentamethylbenzene or the mixture of the biphenyl ether and magnolol were unsuccessful. The lack of induction by PMB might be interpreted as evidence of the absence of P-450 involvement in magnolol and biphenyl ether metabolism in unadapted species, or simply as failure of a synthetic inducer to induce P-450 isozymes with the same specificity as the toxin itself (Berenbaum et al., 1991). Nonetheless, the lack of induction by magnolol and biphenyl ether mixture suggests that detoxification activity in *C. promethea*, by whatever mechanism is present, is relatively modest and not inducible. Whether or not the P-450-like activity in *C. securifera* is constitutive

or induced by sweetbay constituents is unclear, but it appears to be qualitatively and quantitatively different from the detoxification activity in *C. promethea*.

An important component of this study was the comparison of generalist and specialist species within a phylogenetic lineage, since there are relatively few plant–herbivore systems in which both ecologically limiting phytochemicals and the detoxification mechanisms for dealing with them have been characterized among related herbivore species. The finding that biphenyl ether metabolism, although apparently due to cytochrome P-450-like enzymes, is not positively correlated with diet breath across the three taxa contributes to the growing body of evidence that detoxification enzymes in herbivores are more closely correlated with the specific chemistry of their hosts than with diet breadth per se (Nitao, 1989; Berenbaum, 1991). Such substrate specificity, compounded by the phenotypic plasticity exhibited by many enzymatic systems, adds to the already substantial methodological challenges of quantifying metabolic costs that might be associated with different detoxification systems in herbivores (Neal, 1987).

Although the inability to metabolize toxic sweetbay phytochemicals provides a reasonable proximal explanation for patterns of differential host use observed among *Callosamia* species, the evolutionary origin of sweetbay feeding in this group remains unclear. The use of magnoliaceous hosts is not unique to the genus *Callosamia* among the New World Attacini (Saturniidae), for example, *Samia cynthia* has been reported to feed on tuliptree, and there are occasional reports of polyphagous species such as *Hyalophora cecropia* and *Anthaerea polyphemus* using tuliptree (Stone, 1991). However, *Callosamia* is the only group in which all members routinely use magnoliaceous hosts, and *C. securifera* is the only saturniid that uses sweetbay as a host. It should be noted that neither biphenyl ether nor magnolol is found in tuliptree, the magnoliaceous host most frequently used by other attacine moths (Johnson, unpublished data).

Even within the *Callosamia* group, the phylogenetic relationship of sweetbay feeding is unclear, because it is not clear whether *C. securifera* occupies a more basal or derived position within the genus. *Callosamia* is considered to be a compact and discrete group that is well-differentiated from *Hyalophora* and *Eupackardia* (Ferguson, 1972), and the fact that interspecific hybrids can easily be obtained by hand-pairing testifies to the close intrageneric relationships (Piegler, 1977, 1980). Under natural conditions, the species are reproductively isolated by temporal differences in flight and mating times (Ferguson, 1972). Reproductive isolation is apparently complete, as fixed differences at several alleles have been recorded by allozyme electrophoresis (Johnson et al., 1996b). Although *C. angulifera* has been regarded by some as the more primitive member of the genus due to its nocturnal habit, calculations based on allele frequencies show equivalent genetic distances among the three species (Johnson et al., 1996b). This genetic evidence, in combination with the morphological similarities and the potentially rapid nature of the reproductive isolation mechanism, is

consistent with the idea that *C. promethea*, *C. angulifera*, and *C. securifera* may have arisen rapidly and relatively simultaneously from a common ancestor with the ability to feed on magnoliaceous hosts.

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AGE-DEPENDENT EFFECTS OF JASMONIC ACID TREATMENT AND WIND EXPOSURE ON FOLIAR OXIDASE ACTIVITY AND INSECT RESISTANCE IN TOMATO

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Abstract—Jasmonic acid (JA) treatment of tomato plants induces several defense-related oxidative enzymes and increases pest resistance in a manner thought to simulate natural insect wounding. In a full-factorial greenhouse experiment, we examined the independent and interactive effects of plant age and exposure to wind-induced mechanical stress (MS), on the ability of JA to induce defense in tomato. In general, treatment of 4-, 6-, and 8-week-old tomato plants with 1 mM JA resulted in the induction of peroxidase and polyphenol oxidase activity and reduced the relative growth rate of first-instar *Manduca sexta* larvae fed treated leaves, in accordance with other studies. Peroxidase activity increased with plant age and was induced by JA most strongly in older plants. In contrast, polyphenol oxidase activity did not change with plant age and was induced by JA most strongly in young plants. While relative growth rates of *M. sexta* were lower on older plants overall, JA reduced growth rates most strongly in young plants, in which JA treatment enhanced polyphenol oxidase activity by more than 70%. MS enhanced the activity of peroxidase, but substantially reduced the activity of polyphenol oxidase; the latter most intensely on older plants. *M. sexta* tended to grow more slowly on MS-treated plants, although this effect was not significant. Thus, reduced polyphenol oxidase activity in MS-treated plants did not lead to an increase in growth rate of *M. sexta*, possibly because peroxidase activity was still elevated in MS-treated plants. Significant interactions between JA and MS and three-way interactions were not detected for any variable, although the inductive effects of both JA and MS interacted in complex ways with plant age. Our results indicate that resistance traits in tomato are differentially affected by JA and wind exposure and differ in their relative contribution to defense as plants age.

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Key Words—Tomato, jasmonic acid, mechanical stress, peroxidase, polyphenol oxidase, *Manduca sexta*, tobacco hornworm, induced defense, plant age, wind.

INTRODUCTION

Jasmonic acid (JA) treatment of such plants as tomato induces several classes of defense-related compounds and can increase resistance of plants to a variety of pests (Farmer et al., 1992; Thaler et al., 1996). Because JA appears to function as an endogenous regulator of wound-induced chemistry in plants (Creelman et al., 1992), the manner in which low-level applications of exogenous JA induce defense is thought to simulate natural insect wounding (e.g., Baldwin et al., 1994). For example, in young tomato plants, Thaler et al. (1996) have shown that exogenous applications of JA or its volatile derivative, methyl jasmonate (MJ), stimulate the activity of such defense-related proteins as proteinase inhibitors, peroxidase, and polyphenol oxidase in a manner similar to feeding by *Helicoverpa zea*. Peroxidase and polyphenol oxidase are involved in the generation of reactive oxygen species, quinones, or hydroperoxides in plant cells and in the polymerization of polyphenolics, carbohydrates, and proteins in plant cell walls (see Felton et al., 1989, for discussion). Because the activity of such enzymes can play a defensive role against pests, their induction in plants by various treatments, including JA, is usually accompanied by increased resistance to pests (Felton et al., 1989; Duffey and Felton, 1991; Thaler et al., 1996). Because it appears that the inductive effect of JA on these enzymes adequately simulates natural wounding, the use of JA enables the examination of influences on the induction of defense in plants without the confounding effect of the removal of leaf area or other damage to the plant (Baldwin and Schmelz, 1994).

Abiotic stimuli can alter some of the same defense-related enzymes in plants as exogenous JA with overlapping effects on pest resistance. For example, English-Loeb et al. (1997) have shown that drought stress can enhance the activity of polyphenol oxidase in tomato and affect the quality of the foliage to an insect pest. Cipollini (1997) has shown that wind-induced mechanical stress (MS) can enhance peroxidase and cinnamyl alcohol dehydrogenase activities and lignification in leaves of bean seedlings. While this response has likely evolved to enhance mechanical strength of tissues exposed to MS in the field (Jaffe and Forbes, 1993), the induction of these compounds has the overlapping effect of increasing the resistance of seedlings to a mite and a fungal pest (Cipollini, 1997). While MS has been examined for its growth- and yield-controlling properties in tomato (e.g., Johjima et al., 1992; Garner and Bjorkman, 1996), the effect of wind-induced MS on the induction of defense-related compounds and pest resistance has never been examined in this plant.

In addition to wounding, endogenous JA plays a signaling role in the re-

sponse of plants to touch-induced MS (Weiler, 1993) and to other abiotic stimuli such as ultraviolet light exposure (Conconi et al., 1996). In wheat, wind exposure has been shown to induce lipoxygenase gene expression, which is involved in JA synthesis (Mauch et al., 1997) and is associated with increased resistance to insects (Bi et al., 1994; Thaler et al., 1996). Thus, overlapping effects of MS on pest resistance, such as those seen in bean (Cipollini, 1997), may be partially mediated by increased lipoxygenase and/or JA synthesis, leading to general increases in plant defense traits. The apparent use of JA by plants in the translation of mechanical signals suggests that, in addition to its independent effects, exposure to wind-induced MS may also interact with the induction of defense by exogenous JA. For example, simultaneous wind exposure could either heighten or diminish the inductive effect of exogenous JA. Not surprisingly, the potentially interactive effect of wind on JA-induced defense has never been examined, but could be important in the field. Multifactorial studies incorporating environmental variation are necessary in addressing fundamental hypothesis about environmental influences on the induction of resistance in plants.

It is frequently observed that young plants or tissues are more responsive to elicitors of defense than older plants or tissues, although the inverse can be true (Karban and Myers, 1989; Faeth, 1991). For example, the ability of wounding to induce proteinase inhibitor activity in tomato decreases with plant age (Wolfson and Murdock, 1990). Thipyapong and Steffens (1997) have shown that mRNA of polyphenol oxidase F (the most wound-responsive polyphenol oxidase gene family member) is induced more strongly by wounding or JA in young leaves of tomato than in older leaves on the same plant. Constitutive levels of defense-related enzymes such as peroxidase in noninduced plants can vary with plant age as well (Bruce and West, 1989). To our knowledge, the quantitative effect of plant age on the induction of defense by JA has never been examined statistically. While Thaler et al. (1996) noted that young tomato plants were apparently more inducible by JA than older plants, their design did not allow a statistical examination of interacting effects of age on JA-induced defense. Plant age could also influence the independent and interactive effects of wind-induced MS on defense induction and pest resistance, should they exist.

In a full-factorial greenhouse study, we assessed the independent and interactive effects of JA treatment and wind-induced MS on the activity of peroxidase and polyphenol oxidase in leaves of tomato plants of three different ages. We then examined the effect of these treatments on the relative growth rate of the tobacco hornworm, *Manduca sexta*, fed leaves of the same plants. We found that both JA treatment and wind-induced MS statistically interacted with plant age in complex ways to affect defense traits and biological resistance in tomato. Although wind-induced MS independently affected defense traits in tomato, no interactions between JA and wind-induced MS were seen in this experiment.

METHODS AND MATERIALS

Plant Growth. Tomato plants (*Lycoperiscon esculentum* cv. Burpee Big Boy) were grown from seed in flats of Metro-Mix 250 potting soil in the greenhouse under natural light. At the three- to four-leaf stage, seedlings were transplanted to 1.5-liter pots in Metro-Mix 250 potting soil. Three separate plantings staggered in two-week intervals were conducted to produce plants that were 4, 6, and 8 weeks old at the start of the experiment. During the experiment, maximum midday PAR averaged 1200 μmol photons/m^{-2}/sec and greenhouse temperatures ranged from 25 to 30°C. Seedlings were watered regularly and fertilized with 20-20-20 N-P-K soluble fertilizer approximately every five days.

Experimental Design. Plants 4, 6, and 8 weeks old were randomly assigned to one of the following four treatments: (1) treatment with a solution of 1 mM JA on days 1, 4, and 7 of a nine-day period; (2) treatment with daily 1-hr periods of wind-induced MS at 3 m/sec (6.7 miles/hr) for nine days; (3) treatment with 1 mM JA and MS as above for nine days; or (4) untreated.

The focal leaf used for chemistry and bioassays was the third leaf down from the apex on all plants. This leaf was the same age on all plants, thus isolating the effect of plant age and not leaf age. Only this leaf received JA treatment. Jasmonic acid (Sigma Chemical Co., St. Louis, Missouri) was dissolved in a small amount of ethanol and made up to a concentration of 1 mM with distilled water. JA solution was applied by spraying focal leaves until runoff at approximately 12:00 hr on treatment days. JA spray was confined to the focal leaves by shielding nontreated leaves on the same plant with pieces of cardboard. About 1 ml of JA solution was applied per plant on average, i.e., approximately 120 μg JA to each plant. Smaller plants received slightly less and larger plants slightly more. JA induces defense in a dose-dependent manner in tomato, and this amount and method of application has been shown to induce peroxidase and polyphenol oxidase effectively in four-leaf tomato plants (Thaler et al, 1996). Induction of polyphenol oxidase and proteinase inhibitors in four-leaf plants levels off above treatment with 1 mJ JA, although peroxidase and lipoxygenase activities are induced further by 10 mM JA. The third leaf down from the apex on plants not receiving JA was sprayed with the same ethanolic solution minus JA. JA-treated plants were sprayed in isolation from non-JA-treated plants and were allowed to dry before being returned to the greenhouse benches.

Plants receiving wind-induced MS experienced two 1-hr periods of wind at 3 m/sec at 07:00 and 16:00 hr for nine days. Wind was produced by six 60- × 60-cm electric box fans placed along one edge of the greenhouse benches. Fan placement did not interfere with light reception. The identical wind treatment was shown to induce peroxidase activity, lignification, and resistance to pest in bean leaves (Cipollini, 1997, 1998). This noninjurious treatment largely isolates the mechanical component of wind (seismic stress), while keeping the alteration

of temperature and humidity at a minimum. No wind-induced leaf damage was observed in this experiment, but the appearance of damage increases at wind speeds above this level in the greenhouse (D. Cipollini, personal observation). In plants receiving both wind and JA treatment, JA solution was sprayed at least 3 hr before the next wind treatment occurred. Untreated plants were grown in the same greenhouse room. Plants of all ages and treatments were randomly assigned to positions on the greenhouse benches. During the experiment, plants were moved randomly within benches daily and the fan setup moved among benches three times during the nine-day treatment period. There were six replicate plants in each treatment combination.

Bioassay. Eggs of the tobacco hornworm, *Manduca sexta*, and artificial hornworm diet were obtained from Carolina Biological Supply (Burlington, North Carolina). Larvae were hatched from these eggs and raised on artificial hornworm diet at ~25°C until their use in bioassays. Following the last wind treatment on day 9 of the experiment, the two opposing penultimate leaflets from the focal leaves were detached from each plant with a razor and placed in floral water-picks. One preweighed first-instar *M. sexta* larva was placed on each leaflet and allowed to feed for 48 hr at 25°C. Larvae were then removed, weighed, and relative growth rate (RGR) calculated according to Waldbauer (1968). The mean RGR of the two subsamples from each plant was used in statistical tests. Only three larvae of 144 ate the entire leaflet they were offered. Otherwise no resource limitation occurred that could have influenced the results.

Chemical Analyses. From the same focal leaves used in biossays, the two opposing leaflets immediately below the penultimate leaflets were detached with a razor, flash frozen in liquid nitrogen, and stored at −20°C until analysis for enzyme activity. Leaflets were harvested for chemical analyses at the same time they were harvested for the bioassay. One leaflet was analyzed for peroxidase activity and the other for polyphenol oxidase activity. Stout et al. (1996) have shown that the activity of these two enzymes is very similar among adjacent leaflets within a tomato leaf. Thus, the activity of these enzymes in the leaflets used for chemical analysis was assumed to reflect their activity in the leaflets used for bioassays. Peroxidase and polyphenol oxidase activity were analyzed spectrophotometrically in soluble leaf protein extracts using guaiacol and caffeic acid, respectively, as in Thaler et al. (1996).

Statistical Analyses. The random allocation of plants to treatments and random movement among benches allowed the use of a completely randomized 3 × 2 × 2 full-factorial design. Bioassay and chemical data were analyzed by three-way ANOVA with plant age (4, 6, and 8 weeks old), JA treatment (+ or −), and wind treatment (+ or −) as main effects plus all interaction terms. Means were separated with Tukey's HSD tests. Differences among plant ages are discussed in the text, but mean separations shown in Figure 1 below reflect differences between treatments within each age only. Data from all plants were also exam-

ined with multiple regression to establish the quantitative relationship between peroxidase and polyphenol oxidase activity of each leaf and the RGR of *M. sexta* larvae feeding on that leaf. All data were analyzed using SAS (SAS Institute, Cary, North Carolina).

RESULTS

Bioassay. As expected, RGR of *M. sexta* on leaflets of tomato was reduced by JA treatment (JA: $F_{1,60} = 88.82$, $P < 0.001$) (Figure 1a). RGRs on all plants where JA was added were lower than where JA was not. While RGRs were lower on 6- and 8-week-old plants overall and the effect of age alone was significant (age: $F_{2,60} = 25.48$, $P < 0.001$), the effect of JA was dependent on plant age (JA × age: $F_{2,60} = 9.70$, $P < 0.001$). JA reduced RGRs most intensely on 4-week-old plants (~35%) in which RGRs in the absence of JA were the highest. JA reduced RGRs in 6- and 8-week-old plants to a similar degree. In most plants exposed to wind, RGRs were slightly lower than where wind was absent, but the effect of wind alone was not significant (wind: $F_{1,60} = 2.00$, $P = 0.16$).

Chemical Analyses. In general, peroxidase activity of tomato leaves was enhanced by JA treatment (JA: $F_{1,60} = 19.51$, $P < 0.001$) (Figure 1b). Peroxidase activity also increased with plant age, and the effect of age alone was significant (age: $F_{2,60} = 76.52$, $P < 0.001$). However, the inductive effect of JA on peroxidase activity was dependent on plant age (JA × age: $F_{2,60} = 5.18$, $P < 0.01$). Significant induction of peroxidase activity by JA was not present in 4-week-old plants, but it was evident in 6-week-old plants. Induction of peroxidase activity by JA was most intense in 8-week-old plants (~30%) in which peroxidase activity was constitutively the highest. In all treatments except one, peroxidase activity was enhanced by wind exposure and the overall effect of wind alone was significant (wind: $F_{1,60} = 3.76$, $P = 0.05$).

Polyphenol oxidase activity (Figure 1c) in tomato leaves was enhanced by JA (JA: $F_{1,60} = 70.01$, $P < 0.001$), essentially in the same pattern in all ages of plants. Induction by JA ranged from ~45–65% overall, indicating that polyphenol oxidase activity was more inducible by JA than peroxidase activity, an effect also noted by Thaler et al. (1996). Interestingly, polyphenol oxidase activity was reduced by wind exposure (wind: $F_{1,60} = 11.23$, $P < 0.01$), and this effect was dependent on plant age (wind × age: $F_{2,60} = 3.32$, $P < 0.05$). This effect of wind was not present in 4-week-old plants, but ranged from a 25% reduction in 6-week-old plants to a 45% reduction in 8-week-old plants. Polyphenol oxidase activity tended to be lower in 4- and 8-week-old plants, but the effect of plant age alone only approached significance (age: $F_{2,60} = 2.63$, $P = 0.08$).

Quantitative Relationship Between Peroxidase and Polyphenol Oxidase Activity and RGR of M. sexta. Multiple regression of RGR of *M. sexta* on the per-

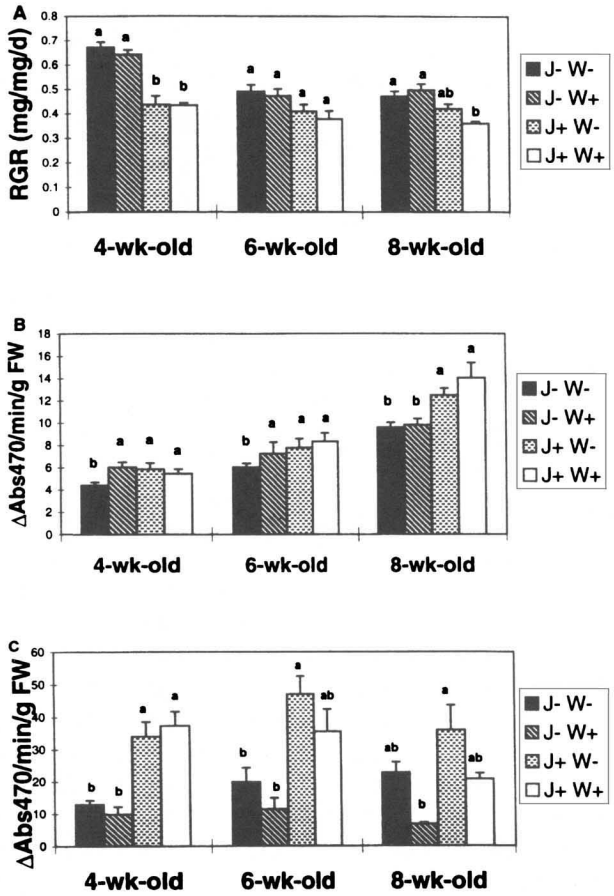

FIG. 1. (A) Relative growth rate (±SE) of first-instar *M. sexta* larvae on leaves of 4-, 6-, and 8-week-old tomato plants in a 48-hr detached-leaf biossay after nine days of treatment. (B) Peroxidase activity (±SE) in leaves of the same plants. (C) Polyphenol oxidase activity (±SE) in leaves of the same plants. Within each age on each figure, bars with different letters are significantly different at $\alpha = 0.05$. Treatments: J– W– = no JA, no wind; J– W+ = no JA, plus wind; J+ W– = plus JA, no wind; J+ W+ = plus JA, plus wind.

oxidase and polyphenol oxidase activity over all samples yielded the following model:

$$\text{RGR of } M.\ sexta = -0.0022\ (\text{peroxidase activity}) - 0.0026\ (\text{polyphenol oxidase activity}) + 0.645$$

This negative relationship was significant ($F_{2,69} = 17.01$, $P < 0.001$; $R^2 = 0.331$). Significant negative relationships were also obtained when RGR was regressed on peroxidase activity and polyphenol oxidase activity separately over all samples. The activity of each enzyme separately explained an equal amount of the variation in RGR of *M. sexta* (data not shown).

DISCUSSION

As expected, treatment with 1 mM JA was shown to be an effective elicitor of both peroxidase and polyphenol oxidase activity in leaves of tomato plants and to slow the RGR of first-instar *M. sexta* fed the same leaves. This finding is in general accordance with other studies (e.g., Thaler et al., 1996). Polyphenol oxidase activity was more responsive to JA treatment than peroxidase activity, also consistent with Thaler et al. (1996) and Stout et al. (1998). While wind alone had only a slight and nonsignificant effect in reducing RGR of *M. sexta*, it slightly, but significantly increased the activity of peroxidase and greatly reduced the activity of polyphenol oxidase. The finding that wind-induced MS reduced the activity of polyphenol oxidase activity was surprising, given that most phenol oxidizing enzymes studied to date in response to MS (such as peroxidase and cinnamyl alcohol dehydrogenase) are induced by MS treatment (DeJaegher et al., 1985; Cipollini, 1997, 1998). The fact that RGRs of *M. sexta* were still slightly reduced or not affected on wind-treated plants suggests that reduced polyphenol oxidase activity in wind-treated plants did not reduce resistance because elevated peroxidase activity in those leaves was sufficient to maintain resistance. Biological resistance could have been affected by many factors not measured in this study (e.g., proteinase inhibitors), but the strong and statistically significant negative relationship between peroxidase and polyphenol oxidase activity and RGR of *M. sexta* across all samples in this study implicates these enzymes as important resistance factors.

Significant interactive effects of plant age on the induction of defense by JA and wind-induced MS were important in this study. Although JA treatment reduced RGRs of *M. sexta* in all ages of plants, JA most effectively reduced RGRs on 4-week-old plants, in which it induced the activity of polyphenol oxidase by about 70%, but in which it had little effect on peroxidase activity. In contrast, JA most strongly induced the activity of peroxidase in 8-week-old plants in which it had the weakest effect on the induction of polyphenol oxidase. While it is known that inducibility of polyphenol oxidase F mRNA decreases with increasing leaf age within one tomato plant (i.e., leaf nodes 1–3, versus leaf node 5 or 7) (Thipyapong and Steffens, 1997), this is the first demonstration that inducibility of polyphenol oxidase activity decreases in leaves of the same age from the same relative position with increasing plant age. While wind had lit-

tle effect on RGR of *M. sexta*, it increasingly reduced the activity of polyphenol oxidase with increasing plant age. Over all treatments, the activity of polyphenol oxidase explained the variation in RGR of *M. sexta* most strongly in 4-week-old plants, but the activity of peroxidase explained the variation in RGRs of *M. sexta* most strongly in 8-week-old plants.

This finding suggests that the contribution of certain defense traits to resistance in tomato may change with plant age. As peroxidase activity increases constitutively with age, its inducibility and importance to plant defense may increase. In turn, because polyphenol oxidase activity did not change as much with plant age, and its inducibility declined, its importance in plant defense may decline with plant age. In a study using young tomato plants, Wolfson and Murdock (1990) showed that the induction of proteinase inhibitors by artificial wounding declined with plant age. However, the ability of wounding to reduce the growth of *M. sexta* larvae in their study did not show a concomitant decline with age, suggesting that other defense traits (peroxidase activity?) became more important as plants aged. The finding that wind-induced MS increasingly reduced the activity of polyphenol oxidase with plant age suggests that polyphenol oxidase activity becomes less reliable as a defense trait in the face of an additional environmental stress as plants mature. It is often thought that the inducibility of plants should decline with age as levels of constitutive defense compounds increase and relative nutrient and water contents decrease (Karban and Myers, 1989). These results illustrate that inducibility of polyphenol oxidase by JA does appear to follow that pattern, but that inducibility of peroxidase does not.

Although wind-induced MS alone had a slight reducing effect on RGRs of *M. sexta* and had significant and differential effects on enzyme activities, it did not interfere with the ability of JA to induce resistance. Because wind-induced MS did reduce the activity of polyphenol oxidase in treatments where JA was added, it may have dampened the effect of JA on inducing resistance, but no statistical interactions between JA and wind-induced MS on biological resistance were seen. In the field, wind at 3 m/sec (6.7 miles/hr) is generally classified as a light to gentle breeze according to the Beaufort Scale of Wind Force. In bean, the magnitude of the induction of peroxidase activity by wind-induced MS increases quantitatively with wind speed (Cipollini, 1998). Thus, higher wind speeds or longer wind durations, that would be common in the field, could have more pronounced effects on resistance alone or could more strongly interact with JA-induced resistance. Although JA has been shown to induce defense in tomatoes grown in the field where wind is ubiquitous (Thaler et al., 1996), this is the first study to isolate the independent effect of wind on the induction of defense in tomato and to statistically examine its interactive effects with JA and plant age. These results illustrate, however, that increased resistance can still be induced by JA even when simultaneous environmental stimuli greatly reduce the activity of such apparently important defense proteins as polyphenol oxidase (Stout et al., 1998).

In this study, plant age was shown to have important independent and interactive effects on the induction of resistance in tomato by JA and wind-induced MS. The activities of peroxidase and polyphenol oxidase were affected differently by age, JA, and wind treatment, and varied in their contribution to biological resistance with age. Wind-induced MS differentially affected enzyme activities and interacted with plant age in its effects, but had little effect on resistance to *M. sexta* alone. Chronic wind exposure or exposure to higher wind speeds could have more pronounced effects, however. More studies incorporating environmental variation (e.g., wind, nutrients, moisture) should be conducted to fully investigate environmental constraints on the induction of defense by JA, particularly if JA is to be used to stimulate resistance of plants in the field (e.g., Thaler et al., 1996).

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EFFECTS OF SEQUESTERED IRIDOID GLYCOSIDES ON PREY CHOICE OF THE PRAIRIE WOLF SPIDER, *Lycosa carolinensis*

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Abstract—Specialist insect herbivores that sequester allelochemicals from their host plants may be unpalatable to potential predators. However, the host-plant species used may determine the degree of palatability. Spiders, including members of the family Lycosidae, are important predators of invertebrate prey. We fed buckeye caterpillars, *Junonia coenia* (Nymphalidae), reared on *Plantago lanceolata* (containing high levels of iridoid glycosides) or *P. major* (containing low levels of iridoid glycosides) to prairie wolf spiders, *Lycosa carolinensis* (Lycosidae), to determine whether the spiders found insects that sequester iridoid glycosides unpalatable. In a field experiment, spiders ate caterpillars reared on *P. major* significantly more often than caterpillars reared on *P. lanceolata*, although they attacked equal numbers of both types of prey. Spiders that bit caterpillars behind their heads or along the middle of their backs prevented caterpillars from implementing deterrent defensive strategies such as regurgitating or defecating. In a laboratory experiment, we presented spiders with *P. lanceolata*-reared and *P. major*-reared caterpillars simultaneously for eight consecutive trials. Spiders consumed *P. major*-reared buckeyes significantly more often than *P. lanceolata*-reared caterpillars. We found no evidence that the spiders learned to avoid the unpalatable prey.

Key Words—*Junonia coenia*, *Lycosa carolinensis*, iridoid glycosides, spiders, tritrophic interactions, unpalatability.

INTRODUCTION

Specialist insect herbivores that restrict their feeding to one or a few closely related species of host plants may improve their defensive capabilities against

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potential predators (Bernays, 1988; Bernays and Cornelius, 1989; Dyer and Floyd, 1993; Dyer, 1995). This is particularly the case when a host plant contains allelochemicals that may be sequestered by the herbivore, making them unpalatable (Brower, 1984; Dyer, 1995). Host-plant allelochemical content, and thus potential protection for the herbivore, may vary between closely related plant species (Malcolm, 1991) as well as among populations or individuals within a species (Krischik and Denno, 1983; Rowell-Rahier and Pasteels, 1991). For example, monarch butterflies (*Danaus plexippus*, Nymphalidae) vary substantially in palatability depending on what milkweed species they feed on as larvae (Brower, 1984; Malcolm, 1991), and checkerspot butterflies in the genus *Euphydryas* show similar variation in palatability depending on larval host plant (Bowers, 1980, 1981; Bowers and Farley, 1990; Belofsky et al., 1989).

One group of allelochemicals that is important in the chemical defense of a variety of different specialist insect species are the iridoid glycosides (reviewed in Rimpler, 1991). Insects in at least four different orders (Hymenoptera, Lepidoptera, Coleoptera, Homoptera) sequester iridoid glycosides (Bowers, 1988; Bowers et al., 1993; Nishida and Fukami, 1989; Rimpler, 1991). These compounds are terpene-derived molecules, found in over 50 families of plants and produced via the mevalonic acid pathway (Jensen, 1991). Some iridoids are very bitter (Bobbitt and Segebarth, 1969), and this may account for their role in the unpalatability of insects that sequester them.

Spiders are dietary generalists whose importance as predators of herbivorous insects has received increasing attention (Freed, 1984; Riechert and Lockley, 1984; Riechert, 1993). Although few studies have considered spider response to prey that have sequestered host-plant allelochemicals, those that have suggest that spiders may avoid unpalatable prey. For example, desert web-spinning grassland spiders, *Agelenopsis aperta* (Agelenidae), that attacked unpalatable Lepidoptera and Coleoptera rubbed their chelicerae and fangs on grass following encounters with such prey (Riechert, 1981). *Nephila clavipes* (Araneidae), an orb-weaving spider, cuts unpalatable prey from the web (Vasconcellos-Neto and Lewisohn, 1984). Although Nentwig (1983, 1986b) found that certain spider species ate poisonous insects, his poisonous insects consisted mainly of insects that had bites, stings, or noxious odors, not prey that had sequestered host-plant allelochemicals.

The prairie grassland spider, *Lycosa carolinensis* Walckenaer (Lycosidae), is an important invertebrate predator of arachnids and insects (Shook, 1978), as are other lycosids (Edgar, 1969; Hallander, 1970; Punzo, 1991; Riechert, 1993). *Lycosa carolinensis* females reach lengths of 22–35 mm during their more than 3-year lifespan, and the species is found throughout the United States (Farley and Shear, 1973; Shook, 1978). These spiders inhabit burrows during the day for protection against high temperatures (Moeur and Eriksen, 1972), desiccation (Punzo and Jellies, 1983), and predators (Gwynn, 1979). *Lycosa carolinensis*

is a sit-and-wait forager that hunts at night (Kuenzler, 1958; Farley and Shear, 1973). These spiders usually remain stationary until a prey item stumbles upon them, and they then orient toward and pounce upon the prey (Rovner, 1980). The body size of potential prey has been argued to be the main limiting factor governing lycosid prey choice (Anderson, 1974; Nentwig, 1986a). In the experiments described below, we test whether the preys' ability to sequester allelochemicals from their host plants affects the prey choice of lycosid spiders.

To determine how *Lycosa carolinensis* responded to prey that sequestered iridoid glycosides, we reared larvae of buckeye butterfly, *Junonia coenia* Hübner (Nymphalidae), on *Plantago lanceolata* and *P. major* (Plantaginaceae). *Plantago lanceolata* contains two iridoid glycosides, aucubin and catalpol, in amounts as high as 10–12% dry weight (Bowers and Stamp, 1992, 1993); *P. major* contains only aucubin (Duff et al., 1965; Massa and Bowers, unpublished data). Buckeye caterpillars are able to utilize both *P. lanceolata* and *P. major* as host plants as well as several other plant species that contain iridoid glycosides (Bowers, 1984; Scott, 1986). *Junonia coenia* sequesters both aucubin and catalpol (Bowers and Collinge, 1992). When *J. coenia* larvae are fed on *P. lanceolata*, they contain 20–25% dry weight aucubin and catalpol; however, when fed on *P. major*, they contain only aucubin and in a much lower concentration, about 4–5% dry weight (Massa and Bowers, unpublished data).

Junonia coenia occurs throughout the southern United States and coastal California, and in some years may occur as far north as the Canadian border (Scott, 1986). In nature, *Lycosa carolinensis* could contact buckeyes when caterpillars leave defoliated host plants or drop off host plants in a defensive response. In addition, *L. carolinensis* may contact other species of arthropods that also sequester iridoid glycosides. We set up two experiments, one in the field and one in the laboratory, to test how *L. carolinensis* responded to *J. coenia* caterpillars that sequestered iridoid glycosides and to compare their response to caterpillars reared on *P. lanceolata* or *P. major*.

METHODS AND MATERIALS

Field Site and Spider Collection. Lycosa carolinensis used for these experiments were collected from a dry, mixed-grass prairie in Boulder County, Colorado. Voucher specimens are deposited at the University of Colorado Museum, Boulder, Colorado. The field site was on a sloping, north-facing hill that bottomed out at a small creek. The clay–loam soil supported several dominant grasses, *Agropyron smithii*, *Bouteloua gracilis*, and *Stipa viridula*, plus several herb species including *Aster falcatus*, *Gutierrezia sarothrae*, *Liatrus punctata*, and *Ratibida columnifera* (Collinge, 1995).

We collected 25 adult and late-instar *Lycosa carolinensis* from the field site

for the lab experiment by enticing them out of their burrows with simulated prey rustling atop their burrows and turrets. We placed each spider in a 5-gallon aquarium filled with a soil mixture 5 in. deep consisting of two parts potting soil to one part sand. Rocks and straw were placed on the soil surface to imitate native prairie ground litter. Water was provided by cotton-plugged test tubes filled with water placed in the aquarium (Moeur and Eriksen, 1972). We covered the tops of the aquaria with fine netting to prevent escape. The spiders were housed at room temperature in front of large windows to ensure a normal light–dark cycle.

Caterpillar Rearing. Junonia coenia caterpillars were raised in growth chambers with a photoperiod of 14L : 10D and temperatures of 26°C day and 20°C night. *Junonia coenia* larvae were reared from eggs on either *P. major* or *P. lanceolata* leaves, which were picked from areas around campus. Gas chromatographic analysis of larvae showed that those raised on *P. major* averaged 5% aucubin, while those raised on *P. lanceolata* averaged 14% aucubin plus 6% catalpol (dry weight) (Bowers and Massa, unpublished data).

Field Experiment. The field portion of this study was conducted during times of major spider activity from shortly after dusk (21:30 hr Mountain Standard Time) until 01:00 or 02:00 hr, when lycosid activity declined. Trials took place on July 28, August 2, and August 9, 1994. We located spiders by using a headlamp, which caused the spiders eyes to reflect light (Farley and Shear, 1973). These field trials were no-choice trials, in which we slowly approached the spiders and offered them a buckeye caterpillar raised on either *P. lanceolata* or *P. major*. The type of caterpillar offered was determined randomly. We dropped the caterpillars in front of the spiders to gain their attention. Only female spiders were tested and individuals were tested only once. Results of each trial were read into a hand-held taperecorder.

Spiders were given a few minutes to pounce on the prey items, but observations continued until we reached a verdict on the acceptance or rejection of the prey item. We scored an acceptance when the spider continued to hold the prey item for several minutes. A rejection was scored if the spider released the prey. We also noted the behavior of the spiders during capture and acceptance or rejection. A contingency table was used to test the null hypothesis that spiders show no preference between caterpillars raised on the two diets.

Laboratory Experiment. This experiment was conducted from July 12 to August 5, 1994, with 25 spiders and eight trials per spider. Every three days, two fifth-instar buckeye caterpillars, one raised on *P. lanceolata* and one raised on *P. major*, were simultaneously offered to the spiders. The caterpillars were visually identical and were matched for size by measuring their lengths. Each caterpillar was tethered with a 12-in.-long thread of nylon fishing line and anchored to the aquarium side with tape so they were able to freely move around the aquarium. The caterpillars were tied loosely enough so that their defensive responses, such as biting and regurgitating, were not impeded. The string holding the *P.*

major caterpillar was thicker to enable us to distinguish between the two types of caterpillars. The caterpillars were left in the aquarium overnight, and the next morning their fate was recorded. After every two trials, the spiders were given either crickets or grasshoppers to vary their diet (Uetz et al., 1992).

To compare the behavior of spiders towards the two types of caterpillars, we used a palatability index to score the response of spiders to prey as follows: eaten = 2, partly eaten = 1, uneaten = 0 (modified from Ritland, 1991a,b). We calculated response scores for each spider for caterpillars raised on each of the two host-plant species by taking the mean of the results of the eight feeding trials. For each spider we also calculated the percent of caterpillars in the two host-plant categories that were eaten and partially eaten. We then compared the response scores for each spider towards each of the caterpillar types with Mann-Whitney U tests. We also performed Spearman rank correlations between trial number and palatability index. If spiders were learning to avoid prey, we would predict a negative correlation between trial number and palatability score.

RESULTS

Field Experiment. Spiders oriented towards and attacked approximately three quarters of the caterpillars offered (*Plantago major* 76.3%, $N = 38$; *P. lanceolata* 75%, $N = 36$). Spiders accepted larvae reared on *P. major* significantly more often than those reared on *P. lanceolata* (Figure 1, $\chi^2 = 5.12$, $P < 0.05$). Trials in which spiders touched, grabbed, or bit caterpillars and subsequently released them were scored as touched and rejected. Spiders attacked and rejected more larvae raised on *P. lanceolata* than larvae raised on *P. major* ($\chi^2 = 5.261$, $P < 0.05$). Trials where spiders ignored the caterpillars were not included in the statistical analysis (Figure 1). Spiders adjacent to their burrows often took the caterpillars inside their burrows, making it difficult to determine the caterpillar's fate. To be conservative, these trials were scored as acceptances.

Observations of spider–caterpillar interactions showed that caterpillars that were rejected during trials often had droplets of hemolymph where the spiders had bitten them (*P. major*, $N = 3$; *P. lanceolata*, $N = 9$). In several trials, caterpillars regurgitated or defecated on the spider's mouthparts after being grabbed; if the caterpillar had been fed on *P. lanceolata*, they were quickly rejected (six of six). *Junonia coenia* raised on *P. major* implemented similar defensive behaviors, but their excretions were rarely effective deterrents (four of five accepted). Spiders that bit the caterpillars behind the head or along the middle of their backs seemed to prevent the buckeye larvae from maneuvering into defensive position. Five *P. lanceolata* and two *P. major* caterpillars were rejected after being merely touched.

Laboratory Experiment. During the course of the experiment, four of the

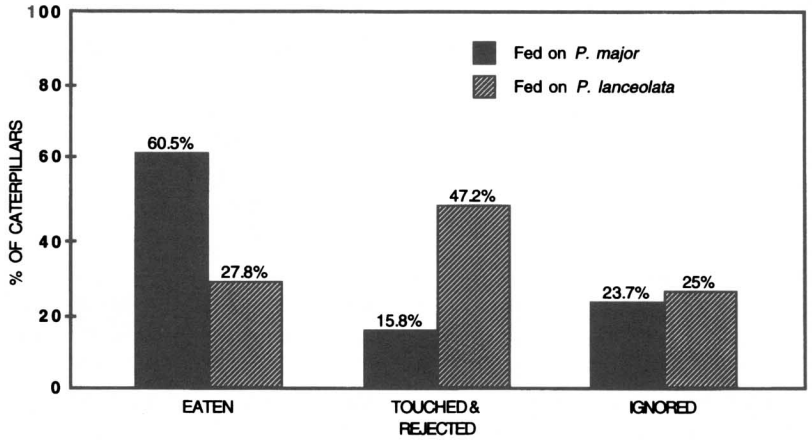

FIG. 1. Comparative acceptability of caterpillars to *Lycosa carolinensis* during field study. The spiders were fed *Junonia coenia* raised on either *Plantago major* (low iridoid diet) or *P. lanceolata* (high iridoid diet) in no-choice trials. Results are presented as percentages for the following categories: caterpillars eaten, caterpillars touched and rejected, and caterpillars that were ignored.

25 spiders died: spiders 5 and 7 died during the first trial, so we did not factor them into the statistical tabulations; spiders 9 and 24 died during intermediate trials and were included in the analysis. Overall we offered a total of 180 pairs of caterpillars to the spiders. The spiders consumed a mean (±SE) of 44.4 ± 3.28% of *P. major* caterpillars ($N = 80$), while only eating 16.7 ± 4.81% of *P. lanceolata* buckeyes ($N = 39$) (Figure 2). When spiders did attack caterpillars reared on *P. lanceolata*, they often abandoned the prey item. These caterpillars were scored as partially consumed (12.2%; $N = 22$). All caterpillars reared on *P. major* were either entirely eaten or left alone. Analysis via Mann-Whitney U tests disclosed that the percentage of caterpillars totally eaten or partially eaten was significantly greater for *P. major*-reared caterpillars ($P < 0.0001$ and $P < 0.0003$, respectively).

The response score (eaten = 2, partly eaten = 1, uneaten = 0) for caterpillars raised on *P. major* was 0.886 ± 0.066, which was significantly higher than the 0.358 ± 0.073 response score for caterpillars raised on *P. lanceolata* (Mann-Whitney U test, $P < 0.0001$), indicating that the spiders ate more of the *P. major*-reared caterpillars.

Spearman rank correlations between trial number and palatability index for each spider were quite variable (Table 1). A Spearman rank correlation statistic is significant at the $P = 0.05$ level if the value exceeds 0.643. A significant negative correlation suggests that the spider was learning to avoid the unpalatable

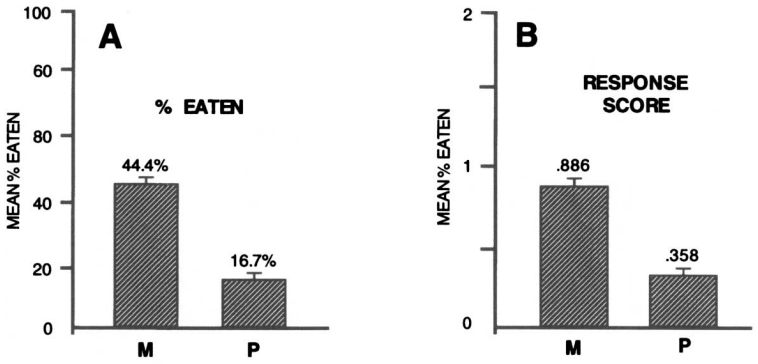

FIG. 2. Comparison of palatability of caterpillars raised on *Plantago major* (M) and *P. lanceolata* (L) that were presented to 25 prairie wolf spiders in the laboratory. Graphs depict: (A) mean percentage entirely eaten and (B) mean response scores where higher values indicate greater palatability.

caterpillars (see Ritland, 1991a). Our results showed that the majority of spiders were not learning to avoid the caterpillars (Table 1), as only two spiders scored correlation coefficient less than −0.643. Of the two spiders that died during the experiment, spider 9 ate every *P. lanceolata* caterpillar offered, while spider 24 consumed increasingly more high iridoid caterpillars (+0.894).

DISCUSSION

Since spiders are generalist predators, can withstand long periods of hunger unless near starvation (Shook, 1978; Punzo, 1991), and can be quite particular about prey acceptance or rejection (Holmberg and Turnbull, 1982; Vasconcellos-Neto and Lewisohn, 1984; Guillebeau and All, 1989; Bardwell and Averill, 1996), we expected *Lycosa carolinensis* to avoid prey with iridoid glycosides. Our results showed that the spiders distinguished between the two types of iridoid glycoside-containing caterpillars, and found *Plantago lanceolata*-reared caterpillars much less palatable than those reared on *P. major*. This was the case in both choice experiments conducted in the laboratory and no-choice experiments conducted in the field. We are unable to say whether the difference in acceptance was due to the amount of iridoid glycosides in the two types of caterpillars (those reared on *P. lanceolata* contained four to five times the amount of those reared on *P. major*), to the fact that those caterpillars reared on *P. lanceolata* contained catalpol as well as aucubin (*P. major*-reared caterpillars contained only aucubin), or to a combination of these factors.

In addition to the iridoid glycosides contained in the body of the caterpillar,

TABLE 1. PERCENT EATEN, RESPONSE SCORES, AND SPEARMAN RANK CORRELATIONS FOR INDIVIDUAL SPIDERS FED *J. coenia* CATERPILLARS REARED ON *P. major* OR *P. lanceolata*[a]

	Eaten (%)		Response score		Spearman correlations	
Spider	*P. major*	*P. lanceolata*	*P. major*	*P. lanceolata*	*P. major*	*P. lanceolata*
1	37.5	12.5	0.750	0.375	0.394	0.546
2	37.5	0.0	0.750	0.000	0.169	0
3	25.0	0.0	0.500	0.125	0.504	0.082
4	37.5	0.0	0.750	0.125	−0.507	−0.082
6	37.5	0.0	0.750	0.125	−0.845	−0.412
8	75.0	25.0	1.500	0.750	0	−0.617
9	28.6	100.0	0.571	0.000	−0.632[b]	0
10	25.0	12.5	0.500	0.250	−0.504	0.082
11	37.5	12.5	0.750	0.625	−0.620	0.496
12	37.5	37.5	0.750	0.750	0.169	−0.732
13	50.0	25.0	1.000	0.625	0	0.206
14	25.0	37.5	0.500	0.875	−0.630	−0.326
15	62.5	0.0	1.250	0.125	0.507	0.082
16	50.0	0.0	1.000	0.125	−0.218	0.412
17	37.5	12.5	0.750	0.125	−0.282	0.247
18	75.0	50.0	1.500	1.375	0	0
19	75.0	0.0	1.500	0.125	0.126	−0.082
20	37.5	12.5	0.750	0.500	−0.282	0
21	62.5	0.0	1.250	0.125	−0.056	0.577
22	37.5	12.5	0.750	0.125	0.169	0.082
23	37.5	12.5	0.750	0.375	−0.169	−0.312
24	40.0	20.0	0.800	0.600	−0.577[c]	0.894
25	50.0	0.0	1.000	0.000	0.218	0
Mean	44.4	16.7	0.886	0.358		
SE	3.28	4.81	0.066	0.073		
Median	37.5	12.5	0.750	0.125		

[a]Each number is the result of eight trials where each spider was offered two caterpillars, one reared on each of the hostplants.
[b]Spider died after seventh trail.
[c]Spider died after fifth trial.

several larvae regurgitated on the spiders when attacked. This regurgitant was an effective deterrent and appeared to be particularly effective when larvae had been reared on *P. lanceolata*. All three *P. lanceolata* caterpillars that vomited on the spiders after being bitten were released. Regurgitation is a potent defense used by many species of lepidopteran and sawfly larvae (Peterson et al., 1987; Gross, 1993); however, it is not always successful at deterring predators. For example, *Ematurga amitaria* (Geometridae) regurgitated in response to attack by lycosid spiders, but 80% of those larvae were killed even after employing this defense (Bardwell and Averill, 1996). The regurgitant from *J. coenia* larvae

does contain iridoid glycosides (Bowers, unpublished data), and this may be the basis of the success of these larvae in using regurgitation as a defense in our experiments.

Some of the spiders used attack strategies that helped them subdue larval prey more successfully. For example, spiders often bit caterpillars behind the head or along their backs, which prevented the larvae from being able to regurgitate on the spiders. These types of behaviors have been documented in other wolf spider species as well (Rovner, 1980).

Buckeye caterpillars containing iridoid glycosides are rejected by a variety of different invertebrate predators. In the present study, we used both laboratory and field experiments to test palatability of buckeye larvae reared on two different host plants to wolf spiders. Other studies have usually been confined to either field or laboratory situations, but all are indicative of the unpalatability of caterpillars that are high in iridoid glycosides. For example, in field studies, two different ant species [*Formica rufa* (Dyer and Bowers, 1996) and *Iridomyrmex humilis* (the Argentine ant) (Camara, 1997)] rejected buckeye larvae that were high in iridoid glycosides. A laboratory study with carpenter ants (*Camponotus floridanus*) also showed that high levels of iridoid glycosides in diets resulted in avoidance by the ants (De la Fuente et al., 1994/1995). Interestingly, in this experiment, there was a difference in how ants behaved depending on whether the trials were in a choice or a no-choice situation: ants would eat diets with higher levels of iridoid glycosides when there was no alternative food available (De la Fuente et al., 1994/1995). In addition, predatory wasps and stinkbugs avoid larvae that have fed on *P. lanceolata* (Stamp, 1992). The wolf spiders in our experiments avoided *P. lanceolata*-reared caterpillars whether in a choice or a no-choice situation. The results of all of these studies indicate that buckeye larvae sequestering high levels of iridoid glycosides are unpalatable to a variety of invertebrate predators.

There is some evidence from the present study and another one, that iridoid glycosides are not only deterrent to spiders, but are toxic. During the course of this study, two spiders died. One of these spiders had eaten every *P. lanceolata*-reared caterpillar it was offered (spider 9, Table 1) and the other had the highest positive correlation between trial number and response score for *P. lanceolata*-reared caterpillars (spider 24, Table 1), indicating that over time, this spider increased its consumption of these caterpillars. In another set of experiments with the jumping spider, *Phidippus audax* (Salticidae), spiderlings fed a diet of *J. coenia* caterpillars had lower consumption and lost weight when fed *J. coenia* caterpillars compared to mealworms (*Tenebrio molitor*, Coleoptera: Carabidae) (Strohmeyer et al., 1998). This was likely the result of both a deterrent effect as well as a physiologically toxic one (Strohmeyer et al., 1998).

Five of the *P. lanceolata*-reared and two of the *P. major*-reared caterpillars offered to spiders in the field were released after being touched but not bitten.

This suggests that there is some cue to which *L. carolinensis* is sensitive through chemical sensors on their legs that occurs externally on the caterpillar (Foelix, 1996). In other studies with spiders as predators, different species of unpalatable larvae, including sawfly larvae, *Pristophorra idiota* (Tenthredinidae) (Bardwell and Averill, 1996), and lepidopteran larvae, *Uresiphita reversalis* (Pyralidae) (Montllor et al., 1991), were rejected without being bitten (Bardwell and Averill, 1996). Deposition of defensive chemicals on or in the cuticle may serve to prevent predators from having to bite larvae to determine that they are unpalatable.

The results of these experiments show that the diet of an oligophagous caterpillar that sequesters defensive chemicals from its host plants (such as *J. coenia*) may be important in determining the outcome of interactions with natural enemies. In our experiments, buckeye caterpillars fed on *P. major* (low in iridoid glycosides) were more often eaten and less often touched and rejected by predatory wolf spiders than those fed on *P. lanceolata* (high in iridoid glycosides). Similar results from other experiments (Stamp, 1992; Dyer and Bowers, 1996; Camara, 1997) indicate that sequestration of iridoid glycosides may be an effective antipredator defense against a diverse assemblage of invertebrate predators. In addition, the host-plant species fed on by larvae and the iridoid glycoside content of the host plant may determine the degree to which an individual caterpillar is protected from potential predators.

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EFFECT OF *Euphorbia esula* ON SHEEP RUMEN MICROBIAL ACTIVITY AND MASS IN VITRO

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Abstract—Most large herbivores avoid the invasive weed leafy spurge (*Euphorbia esula* L.), possibly because the plant contains secondary compounds including terpenoids and condensed tannins. On the other hand, *E. esula* has high nutritive value based on traditional measures such as crude protein, fiber, and dry matter disappearance and is consumed by sheep and goats. Our objective was to determine if material from undefoliated and previously defoliated *E. esula* shoots, mixed in different proportions with grass hay, adversely affects sheep rumen microbial activity and mass in vitro. Material from undefoliated and previously defoliated *E. esula* shoots was collected in June, July, and August 1994 near Grass Range, Montana. Usually, increasing levels of *E. esula* leaves and flowers in the mixtures increased in vitro dry matter and neutral detergent fiber disappearances (DMD, NDFD), microbial gas production, and microbial purine concentrations. In contrast, increasing levels of *E. esula* stems in the mixtures decreased DMD, NDFD, microbial gas production, and microbial purine concentrations. Rumen microbial gas production and purine concentrations were higher with leaves from previously defoliated than undefoliated shoots. In contrast, rumen microbial gas production and DMD were lowest for stems from previously defoliated shoots; these responses correlated with high concentrations of condensed tannins in stems of previously defoliated plants. In early summer, the high nutritive value of *E. esula* appears to offset any potential negative effects associated with secondary compounds. In late summer, microbial response appears more sensitive to the presence of secondary compounds, when nutritive value of this plant is declining.

Key Words—Leafy spurge, terpenes, ruminant, secondary compounds, herbivory, rumen microorganisms, weeds.

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INTRODUCTION

Leafy spurge (*Euphorbia esula* L.), an introduced perennial weed, invades rangelands, displacing native vegetation and reducing rangeland carrying capacity (Reilly and Kaufman, 1979). *Euphorbia esula* infests more than one million hectares in the United States and Canada, primarily in the northern Great Plains of North America (Lajeunesse et al., 1995).

Euphorbia esula tissues and latex contain several secondary plant compounds including terpenoids (Evans and Kinghorn, 1977; Mahlberg, personal communication) and condensed tannins (Roberts and Olson, unpublished data). These secondary compounds deter feeding by many herbivores (Upadhyay et al., 1978; Lym and Kirby, 1987) because they taste bitter or impart a noxious odor. This may explain why cattle consume little, if any, *E. esula* when grazing (Lym et al., 1988). In contrast, domestic sheep will consume *E. esula* until it comprises up to 50% of their daily dry matter intake (Landgraf et al., 1984; Bartz et al., 1985). Goats also consume the plant (Sedivec and Maine, 1993; Walker et al., 1994a).

Although secondary compounds may deter feeding directly, they may also reduce digestion and assimilation by altering rumen function. Terpenoids in essential oils from big sagebrush (*Artemisia tridentata*) inhibit in vitro growth of gram-positive and gram-negative microorganisms taken from the rumens of mule deer (Nagy et al., 1964). Similarly, monoterpene alcohols in Douglas fir (*Pseudotsuga menziesii*) inhibit rumen microbial activity of sheep and deer, reflected by sharp decreases in microbial gas production (Oh et al., 1967).

By reducing rumen function, secondary compounds reduce dry matter digestibility. In vivo crude protein digestibility declines when ponderosa pine (*Pinus ponderosa* Laws.) needles are added to crested wheatgrass [*Agropyron desertorum* (Link) Schultes] hay (Adams et al., 1992). In part, this response was attributed to the effect of phenolics or other secondary compounds on rumen microbial populations. Pine needles had a greater effect on rumen microbial digestion in vitro than in vivo. Similarly, microbial gas production and in vivo dry matter disappearance decreased as concentrations of extractable polyphenols in four Greek browse species increased (Khazaal et al., 1992). Increasing concentrations of phenolics affected microbial gas production more than dry matter disappearance. Because of these results, microbial gas production was considered more accurate than the in vivo nylon bag technique for identifying feeds with antinutritive factors.

Euphorbia esula has high nutritive value based on traditional measures that include crude protein and digestibility (Fox et al., 1991). Despite its high nutritive value, secondary compounds may deter feeding or affect rumen function. Our objective was to determine if material from undefoliated and previously defoliated *E. esula* shoots, when mixed in increasing proportions with grass hay, adversely affects sheep rumen microbial activity and mass. We hypothesized that

increasing levels of plant material from undefoliated and especially defoliated *E. esula* in these *E. esula*–grass hay mixtures would reduce in vitro dry matter disappearance, microbial gas production, and microbial purine concentrations. Microbial gas production was used as an indicator of microbial activity, whereas microbial purine concentration was used as an indicator of microbial mass.

METHODS AND MATERIALS

Field Site and Plant Material. Euphorbia esula was collected from an infested rangeland 17 km southeast of Grass Range, Montana (46°52′N, 108°52′W) in the foothills of the Big Snowy Mountains. Elevation is about 1340 m. Annual precipitation averages 400 mm.

Samples were collected from a large contiguous area in a heavily infested stand to minimize variability that soils on the site may have had on secondary chemistry of *E. esula*. On May 19, June 23, and July 28, 1994, *E. esula* was defoliated to a 3- to 4-cm stubble height on 15-m × 20-m plots. Shoots of *E. esula* were collected on these dates (initial growth). Three weeks later (June 9, July 14, August 18), material from undefoliated shoots and previously defoliated shoots were harvested by hand. Undefoliated shoots were harvested from plots beside previously defoliated plots.

Forage Analysis. All *E. esula* material was air dried. Undefoliated shoots were sorted into leaves, stems, and flowers, if present. Previously defoliated shoots were sorted into leaves and stems. All plant parts were ground to pass through a 1-mm screen in a Wiley mill. Dry matter (DM), ash, crude protein (CP), and ether extract (EE) were determined by standard methods (AOAC, 1984). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were determined by the procedures of Goering and Van Soest (1970). Condensed tannins (CT) were extracted and assayed according to the colorimetric procedure of Burns (1971), as modified by Price et al. (1978).

Separate *E. esula* plant parts were mixed with low-quality grass hay [smooth brome (*Bromus inermis*), 6.3% CP] in the following proportions of *E. esula*–grass hay: 100:0, 75:25, 50:50, 25:75, and 0:100. The grass hay was also ground to pass through a 1-mm screen in a Wiley mill. Forage treatments included these five mixtures *E. esula*–grass hay: 100:0, 75:25, 50:50, 25:75, and 0:100 of the *E. esula* plant parts collected on each of the three sampling dates. Regrowth leaves were not present on previously defoliated shoots collected in August.

Conventional and Modified in Vitro Trials. First, ruminal in vitro dry matter disappearance (IVDMD) was determined for these forage treatments using the first stage of Tilley and Terry's (1963) conventional in vitro system ($N = 3$ runs). Rumen fluid inoculum for in vitro trials was collected from three ruminally fistulated Targhee ewes maintained on a diet of the low-quality grass hay

described previously. Rumen fluid was composited and strained through eight layers of cheese cloth. Forage samples were weighed (0.25 g) into 50-ml fermentation tubes. Twenty milliliters of McDougall's buffer (McDougall, 1948) was added to each tube, and then each tube was inoculated with 5 ml of rumen fluid. Fermentation tubes were flushed with CO_2, capped, and incubated at 39°C. Duplicate fermentation tubes for each forage treatment and blanks were removed from the incubator at 3, 6, 12, and 24-hr postinoculation (Roberts, 1996). Only 24-hr values are reported here. Fermentation tubes were centrifuged at 2000 rpm for 15 min, decanted, and dried at 60°C for 48 hr to determine 24-hr ruminal IVDMD.

Second, we used a modified in vitro system to measure total microbial gas production, purine concentrations, and ruminal IVDMD (Thomas et al., 1994; Olson and Kelsey, 1997). For each forage treatment, duplicate 250-ml flasks were prepared containing 2 g forage, 100 ml McDougall's buffer, and 50 ml strained rumen fluid. In vitro flasks were then placed in a 39°C shaking water bath and gas production was measured by water displacement in inverted burettes. Gas production was measured at 2, 3, 4, 6, 12, and 24 hr (Roberts, 1996). Only cumulative 24-hr values are reported here. Following the 24-hr reading, contents of the flasks were filtered to separate the residue from the fluid fraction. Residues were dried at 60°C for 48 hr and weighed to determine IVDMD. Residues were also analyzed for NDF content (Goering and Van Soest, 1970) and purine concentration of attached bacteria (Zinn and Owens, 1986).

Data Analyses. Twenty-four-hour IVDMD (conventional and modified approaches) was analyzed for each collection date by a general linear model (GLM; SAS, 1987). To adjust for run effect, data were transformed by dividing treatment values by 100% grass hay values for each run. For the modified in vitro trial, 24-hr cumulative gas production, IVDMD, residue NDF fraction, and purine concentrations were analyzed for each collection date with ANOVA. Responses to 100% grass hay were used as covariates.

Planned linear contrasts were used to compare IVDMD (conventional, modified), NDFD, and 24-hr cumulative gas production and microbial purine concentrations from the different plant part (leaves, stems, flowerheads)–collection date combinations (Table 1). Linear, quadratic, and cubic regression coefficients and all possible two- and three-way interactions were used to compare responses to the different mixtures of *E. esula* and grass hay. We also determined correlations between condensed tannin concentrations, microbial gas production, IVDMD, NDFD, and purine concentrations (Jandel Scientific, 1993). $P < 0.10$ are reported (Gill, 1981).

RESULTS

Nutritive Value. The nutritive value of all parts declined as the growing season progressed (Table 2). Regrowth leaves from shoots collected in June and

TABLE 1. PLANNED LINEAR CONTRASTS OF IVDMD (CONVENTIONAL IN VITRO SYSTEM—CON), IVDMD (MODIFIED IN VITRO SYSTEM—MOD), NDFD, MICROBIAL GAS PRODUCTION (MGP), AND MICROBIAL PURINE CONCENTRATIONS (MPC) OF LEAVES (L), FLOWERS (F) AND STEMS (S) COLLECTED FROM UNDEFOLIATED (U) AND PREVIOUSLY DEFOLIATED (PD) *E. esula* IN JUNE, JULY, AND AUGUST 1994

Planned contrasts	L-U vs. S-U	F-U vs. S-U	L-PD vs. S-PD	L-U vs. L-PD	S-U vs. S-PD
IVDMD (CON)					
June	L-U[a]	F-U	L-PD	NS	NS
July	L-U	F-U	L-PD	L-PD	NS
August	L-U	NS	NS	NS	S-U
IVDMD (MOD)					
June	L-U	F-U	L-PD	L-PD	S-PD
July	L-U	F-U	L-PD	L-PD	S-U
August	NS	NS	NS	NS	NS
NDFD					
June	L-U	F-U	L-PD	L-PD	S-PD
July	L-U	F-U	L-PD	L-PD	NS
August	L-U	F-U	L-PD	NS	NS
MGP					
June	L-U	F-U	L-PD	NS	S-PD
July	L-U	F-U	L-PD	L-U	S-U
August	L-U	F-U	L-PD	NS	NS
MPC					
June	L-U	F-U	L-PD	L-PD	NS
July	L-U	F-U	L-PD	L-PD	NS
August	L-U	F-U	L-PD	NS	NS

[a]Plant part indicated in columns 2–6 indicate treatments which had greater response of the treatments contrasted. All $P < 0.001$.

July had higher CP content and lower EE, NDF, ADF and ADL contents than leaves from undefoliated shoots. Crude protein content of *E. esula* leaves collected throughout the summer were higher than CP of grass hay (CP = 6.3%). In contrast, CP content of the grass hay was greater than CP content in *E. esula* stems collected in June, July, and August and flowers from undefoliated shoots collected in August.

Condensed tannins, present in all *E. esula* plant material collected in June, July, and August, increased seasonally except in leaves from previously defoliated shoots (Figure 1). Condensed tannin concentrations were higher in stems from previously defoliated than from undefoliated shoots.

Microbial gas production, IVDMD, NDFD, and microbial purine concentrations were negatively correlated with CT concentrations ($P < 0.04$; Table 3). Dry matter disappearance and microbial gas production were positively correlated (P

TABLE 2. NUTRITIVE VALUES OF *E. esula* PLANT PARTS FROM SHOOTS INITIALLY DEFOLIATED (I) ON MAY 19, JUNE 23, AND JULY 28, AND FROM UNDEFOLIATED PLANTS (U) AND PREVIOUSLY DEFOLIATED PLANTS (PD) HARVESTED 3 WEEKS LATER

Plant part	CP	EE	NDF	ADF	ADL
May 19, 1994					
Leaves-I	22.68	4.18	18.56	15.21	2.94
Flowers-I	25.78	5.02	16.98	14.55	3.49
Stems-I	9.38	3.03	48.01	40.68	5.96
June 9, 1994					
Leaves-U	16.59	4.73	18.45	16.87	3.70
Flowers-U	17.67	5.28	20.74	18.41	4.73
Stems-U	4.85	2.60	63.65	53.64	9.70
Leaves-PD	22.22	4.12	16.61	13.31	2.10
Stems-PD	6.96	3.32	54.39	47.37	8.51
June 23, 1994					
Leaves-I	14.52	4.28	26.20	22.91	5.30
Flowers-I	14.09	4.98	30.16	25.53	6.56
Stems-I	4.22	2.26	65.70	56.39	10.33
July 14, 1994					
Leaves-U	13.27	4.75	25.89	22.96	5.29
Flowers-U	13.23	5.69	31.32	25.59	6.01
Stems-U	4.03	2.13	65.44	56.18	10.48
Leaves-PD	25.73	3.48	18.69	14.10	2.40
Stems-PD	4.08	2.02	71.29	61.99	12.08
July 28, 1994					
Leaves-I	11.14	5.46	30.69	25.65	6.24
Flowers-I	8.65	6.73	36.14	30.45	6.70
Stems-I	3.16	2.48	68.17	57.64	11.62
August 18, 1994					
Leaves-U	8.24	6.55	32.83	28.91	6.36
Flowers-U	6.05	6.73	42.60	36.58	7.64
Stems-U	2.43	2.57	67.98	60.28	11.53
Stems-PD	2.83	2.43	70.38	62.35	12.82

[a]Crude protein (CP); ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL).

= 0.003). Dry matter disappearance was positively correlated with NDFD ($P < 0.0001$) and microbial purine concentrations ($P = 0.003$). Similarly, NDFD was positively correlated with microbial purine concentrations ($P < 0.0001$).

Effect of Plant Part–Collection Date. Based on the conventional and modified approaches, IVDMD of leaves and flowers was higher than IVDMD of stems from undefoliated and previously defoliated shoots collected in June and July (Table 1). Dry matter disappearance of leaves from previously defoliated shoots was higher than of leaves from undefoliated shoots harvested in July. The two approaches gave slightly different results when leaves and stems from

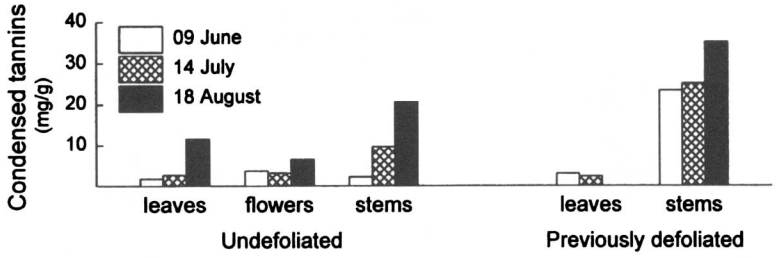

FIG. 1. Concentrations of condensed tannins (mg/g) in plant parts from undefoliated and previously defoliated *E. esula* harvested in June, July, and August 1994.

undefoliated shoots were compared with leaves and stems from previously defoliated shoots. For example, the modified approach indicated stems from previously defoliated shoots collected in June had higher IVDMD than undefoliated stems, whereas the conventional approach did not detect a significant difference.

For material collected on each harvest date, NDFD, microbial gas production, and microbial purine concentrations of leaves and flowers were higher than for stems harvested from undefoliated and previously defoliated shoots (Figures 2–4, Table 1). Leaves from previously defoliated shoots had higher NDFD, microbial gas production, and microbial purine concentrations than leaves from undefoliated shoots. For material collected in June, NDFD and microbial gas production were greater for stems from previously defoliated than from undefoliated shoots. For material collected in June and July, NDFD and microbial purine concentrations were greater for leaves from previously defoliated than from undefoliated shoots. Similar microbial purine concentrations were produced from stems from undefoliated and previously defoliated shoots harvested in June, July, and August.

TABLE 3. CORRELATION COEFFICIENTS OF ASSOCIATIONS OF CONDENSED TANNIN (CT) CONCENTRATIONS, MICROBIAL GAS PRODUCTION (MG), DRY MATTER DISAPPEARANCE (IVDMD), NEUTRAL DETERGENT FIBER DISAPPEARANCE (NDFD), AND MICROBIAL PURINE CONCENTRATIONS (MPC) ($N = 14$)

	Correlation coefficient with			
	CT	MG	IVDMD	NDFD
MG	−0.60			
IVDMD	−0.76	0.73		
NDFD	−0.67	0.35	0.88	
MPC	−0.55	0.19	0.73	0.92

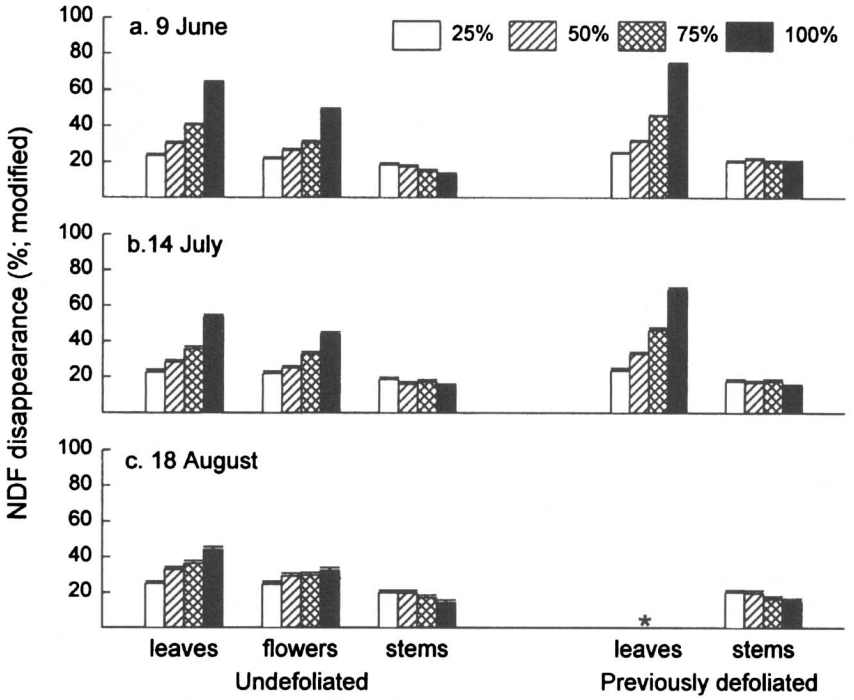

FIG 2. In vitro neutral detergent fiber disappearance (%; 24 hr; modified system) of four mixtures (% *E. esula*) of plant parts from undefoliated and previously defoliated *E. esula* harvested on June 9, July 14, and August 18, 1994. Bars represent least square mean standard errors, although they may be too small to see in some cases.

Effect of E. esula–*Grass Hay Mix.* The conventional and modified approaches resulted in similar, but not identical, IVDMDs (Table 4, Figures 5 and 6). In general, IVDMD of leaves and flowers from undefoliated and previously defoliated shoots harvested in June, July, and August increased as the proportion of *E. esula* in mixtures increased. However, the 75 : 25 mixture of leaves harvested from undefoliated shoots in June had a higher IVDMD than the 100 : 0 mixture with the modified approach (Figure 6). This response was similar to microbial gas production from the different mixtures (Figure 3). In contrast, IVDMD and microbial gas production decreased as the proportion of stems from previously defoliated shoots harvested in July, and stems from undefoliated (IVDMD—conventional only) and prevoiusly defoliated shoots in August increased. Usually, microbial purine concentrations were greatest with the 100 : 0 mixture of leaves and flowers (Figure 4).

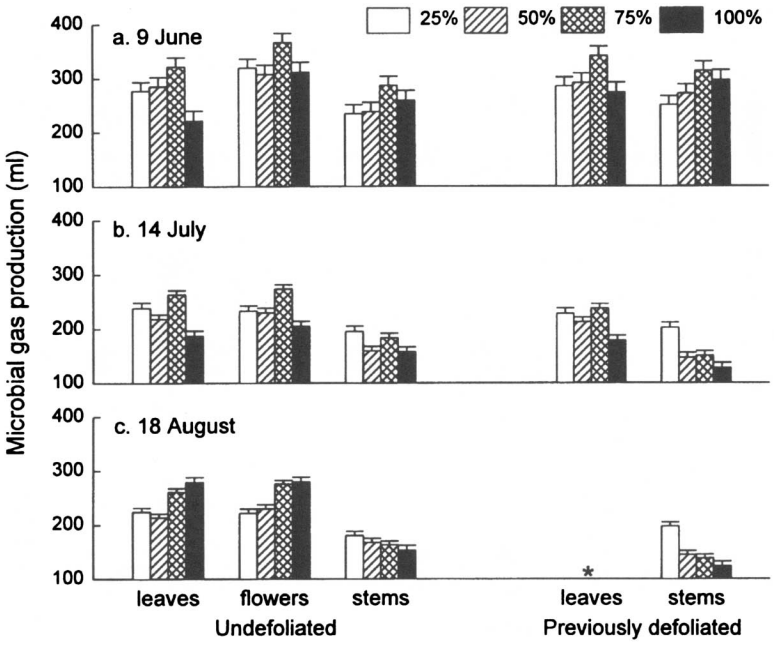

FIG 3. In vitro microbial gas production (ml; 24 hr; modified system) of four mixtures (% *E. esula*) of plant parts from undefoliated and previously defoliated *E. esula* harvested on June 9, July 14, and August 18, 1994. Bars represent least square mean standard errors.

DISCUSSION

Nutritive Value. E. esula is a highly nutritious and digestible forage based on traditional measures of nutritive value including CP, NDF, ADF, and IVDMD. However, *E. esula* contains secondary compounds including condensed tannins and terpenoids (Evans and Kinghorn, 1977; Mahlberg, personal communication). Ruminants may avoid or limit their use of plants containing secondary compounds because the compounds deter herbivory by taste or smell or are toxic to the animal or rumen microbial populations (Striby et al., 1987; Kronberg and Walker, 1993; Provenza, 1995). These factors are not mutually exclusive, and may be intimately related via aversive postingestive feedback (Provenza, 1996).

Condensed tannins were present in leaves, flowers, and stems of *E. esula*. Condensed tannins suppress voluntary forage intake of ruminants by inhibiting microbial growth and activity in the rumen (Smart et al., 1961; Barry and Duncan, 1984) and mammalian enzymes in the small intestines (Kumar and Singh, 1984; Butler et al., 1986; Clausen et al., 1989). High concentrations of CT

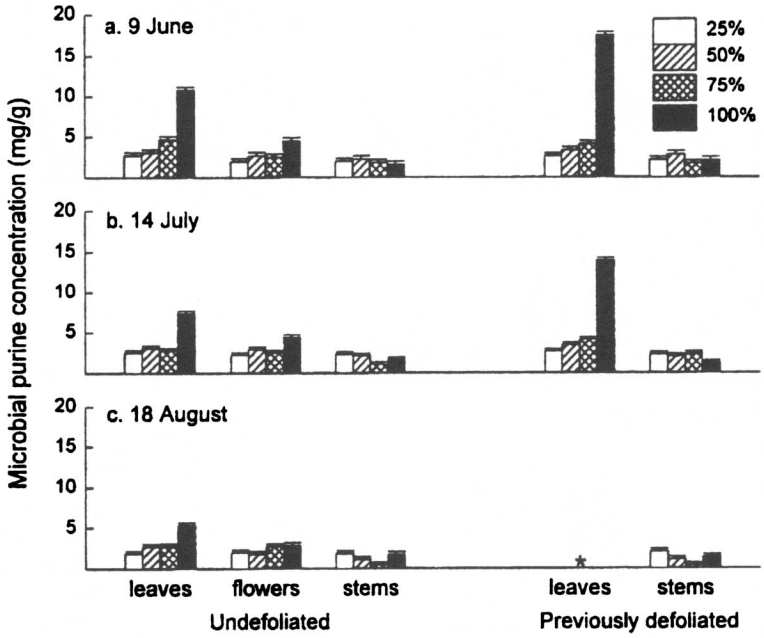

FIG 4. Microbial purine concentrations (mg/g) of four mixtures (% *E. esula*) of plant parts from undefoliated and previously defoliated *E. esula* harvested on June 9, July 14, and August 18, 1994. Bars represent least square mean standard errors, although they may be too small to see in some cases.

in grasslands Maku lotus (*Lotus pedunculatus* L.) depress rumen carbohydrate digestion and voluntary intake in sheep (Barry and Duncan, 1984). Similarly, condensed tannins inhibit cellulose digestion by the ruminal bacteria *Fibrobacter succinogenes* (Bae et al., 1993).

Rumen fiber digestion in sheep is depressed when CT concentration in the diet is about 4% DM, whereas intake and growth are depressed when CT concentrations range from 6 to 10% DM (Barry and Duncan, 1984; Barry, 1985; Barry et al., 1986). Voluntary intake by kudus, impalas, and goats decreases when CT concentrations are above 5% DM in leaves of certain South African plants (Cooper and Owen-Smith, 1985). In our study, CT concentrations in previously defoliated *E. esula* stems approached these levels, especially in late summer. Condensed tannin concentrations were negatively correlated with IVDMD, NDFD, microbial gas production, and microbial purine concentrations. This would explain why high CT concentrations often slow passage rates and thus limit voluntary intake.

Concentrations of CT increased in *E. esula* stems after the plant was defo-

TABLE 4. EFFECT OF *E. esula*–GRASS HAY MIXTURE ON IVDMD (CONVENTIONAL IN VITRO SYSTEM—CON), IVDMD (MODIFIED IN VITRO SYSTEM—MOD), AND MICROBIAL GAS PRODUCTION OF LEAVES (L), FLOWERS (F), AND STEMS (S) COLLECTED FROM UNDEFOLIATED (U) AND PREVIOUSLY DEFOLIATED (PD) *E. esula* IN JUNE, JULY, AND AUGUST 1994[a]

Plant Parts	L-U	F-U	S-U	L-PD	S-PD
IVDMD (CON)					
June	l[b] (100:0)	l (100:0)	NS[c]	l (100:0)	NS
July	l (100:0)	l (100:0)	NS	l (100:0)	l (0:100)
August	l (100:0)	l (100:0)	l (0:100)	l (100:0)	l (0:100)
IVDMD (MOD)					
June	q (75:25)	l (100:0)	NS	l (100:0)	NS
July	q (75:25)	l (100:0)	NS	l (100:0)	l (0:100)
August	l (100:0)	l (100:0)	NS	l (100:0)	l (0:100)
MGP					
June	q (75:25)	NS	NS	q (75:25)	NS
July	q (75:25)	q (75:25)	NS	NS	l (0:100)
August	l (100:0)	l (100:0)	l (0:100)	NS	l (0:100)

[a]Ratios in parnetheses indicate *E. esula*–grass hay mixture with greatest IVDMD and microbial gas production.
[b]Mixtures were tested for linear (l), quadratic (q), or cubic (c) responses ($P < 0.05$). The most significant response was used when two or more responses were less than 0.05.
[c]NS indicates no significant ($P > 0.05$) linear, quadratic, or cubic response.

liated. Defoliation-induced increases in CT have been observed in other forage species, from blackbrush (*Coleogyne ramosissima*; Provenza and Malecheck, 1984) to Douglas fir trees (*Pseudostuga menziesii*; Walters and Stafford, 1984). In contrast, clipping or browsing did not induce the production of CT in several Alaskan and Mediterranean woodland species (Chapin et al., 1985; Perevolotsky, 1994).

In June, CT concentrations were highest in flowers of *E. esula*. However, in July and August, CT concentrations were highest in stems. Condensed tannin concentrations often differ among specific plant parts (e.g., Terrill et al., 1992). Flowers may contain higher concentrations of CT early in the growing season to defend developing reproductive tissues.

Condensed tannin concentrations increased seasonally in all *E. esula* plant parts. Similarly, CT concentrations increase in sericea (*Sericea lespedeza*), birdsfoot trefoil (*Lotus corniculatus*), and big trefoil (*Lotus uliginosus*) as these plants mature (Burns, 1966; Cope et al., 1971; Chiquette et al., 1989; Lees et al., 1994). Early in the growing season, *E. esula* may allocate more carbohydrates to growth and reproduction than to producing tannins, which are carbon-based compounds with high metabolic costs (Skogsmyr and Fagerström, 1992).

Terpenoids in *E. esula* may also affect rumen function. For example, vari-

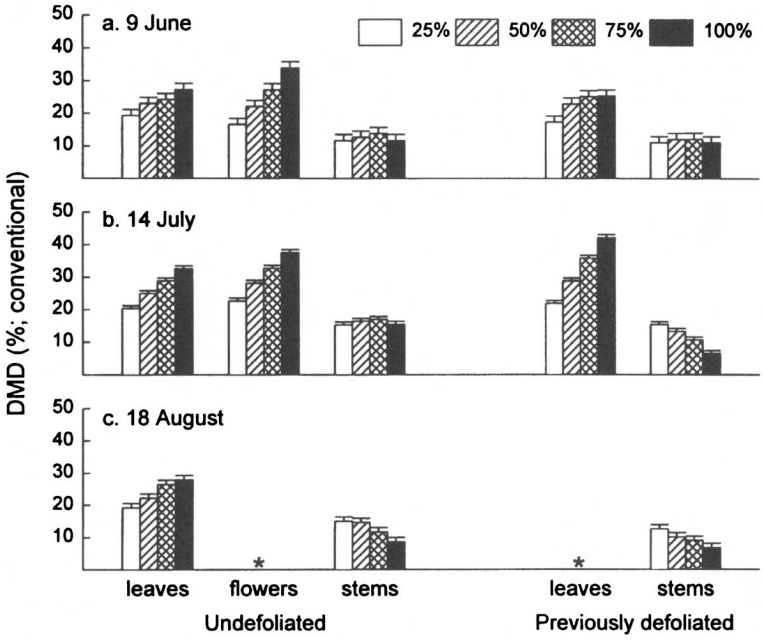

FIG. 5. In vitro dry matter disappearance (%; 24 hr; conventional system) of four mixtures (% *E. esula*) of plant parts from undefoliated and previously defoliated *E. esula* harvested on June 9, July 14, and August 18, 1994. Bars represent least square mean standard errors, although they may be too small to see in some cases.

ous diterpenoid ingenols have been found in *E. esula*. Their activities range from inflammatory agents to weak tumor-promoting properties (e.g., Kupchan et al., 1976; Upadhyay et al., 1978; Seip and Hecker, 1982). However, sheep were not averted to a novel food when its consumption was followed by an intravenous injection of ingenol (Kronberg and Walker, 1993). They were averted when the consumption of the novel food was followed by a dose of *E. esula* fermented with sheep ruminal digesta. This suggests that ingenol alone is not aversive. In another study, *E. esula* elicited food aversions in rats (Kronberg et al., 1995); an acetone extract of *E. esula*, presumably containing ingenol esters and other secondary compounds, elicited food aversions in sheep and rats. These studies indicate that ingenol in *E. esula* may be only one of several secondary compounds, including CT, that elicits food aversions in large ruminants.

The presence of secondary compounds may explain why cattle avoid *E. esula*. Usually generalist grazers, including domestic cattle, have low levels of secondary compounds in their diet and cannot metabolize these compounds. Conversely, specialist grazers and browsers typically consume forages with high

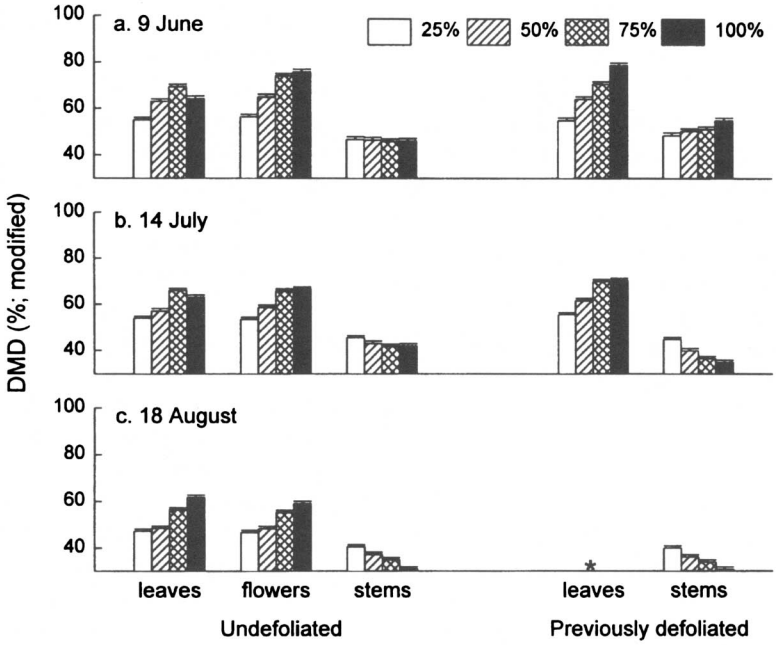

FIG. 6. In vitro dry matter disappearance (%; 24 hr; modified system) of four mixtures (% *E. esula*) of plant parts from undefoliated and previously defoliated *E. esula* harvested on June 4, July 14, and August 18, 1994. Bars represent least square mean standard errors, although they may be too small to see in some cases.

concentrations of secondary compounds. Sheep, goats, and mule deer produce high-proline tannin-binding proteins in their saliva, allowing them to use forages containing condensed tannins (Robbins et al., 1987; Austin et al., 1989; Mehanso et al., 1992). Cattle do not produce these salivary proteins (Jones and Mangan, 1977; Austin et al., 1989).

Microbial populations in different ruminant species may differ in their ability to degrade secondary compounds (Wachenheim et al., 1992). For example, five ruminal bacteria species varied in the amount of tannins bound and the amount of tannin required to inhibit their growth (Nelson et al., 1997). Sheep were averted to a novel food when its intake was followed by a dose of *E. esula* fermented with sheep ruminal digesta, but not when followed by a dose of *E. esula* fermented with goat ruminal digesta (Kronberg and Walker, 1993). This suggests that goat ruminal microbial populations differ from those of sheep in their ability to tolerate or detoxify certain secondary compounds in *E. esula*.

In Vitro Microbial Activity and IVDMD. Leaves and flowers of *E. esula* usually had higher in vitro microbial activity and IVDMD than stems. This could

be attributed to the greater levels of CP and EE, and lower levels of NDF, ADF, ADLN, and CT in leaves and flowers compared with stems.

Trends in IVDMD were similar for the conventional and modified in vitro systems. However, 24-hr IVDMD values for all plant parts and mixtures from the modified in vitro system were about two times higher than those for the conventional in vitro system. Flasks in the modified system were inoculated with twice as much rumen fluid as those in the conventional system. In addition, residues from the modified system were rinsed, accounting for slightly higher IVDMD values than those from the conventional system. Furthermore, shaking the flasks in the warm water bath with the modified approach may more closely simulate conditions in the rumen compared with the "still" conditions under the conventional approach.

Effect of Plant Part–Collection Date and E. esula*–Grass Hay Mix.* Mixing different plant parts of *E. esula* with grass hay often resulted in associative effects. These are nonlinear responses in nutrient utilization by combining two feedstuffs in ruminants diets (Moe, 1979; Hassan et al., 1988). Positive associative effects may result from increased microbial activity, whereas negative associative effects may result from reduced rates of fiber digestion (Hart, 1987). For example, adding increasing amounts of *E. esula* leaves and flowers to grass hay increased IVDMD, which may reflect the higher CP and lower NDF content of leaves and flowers from *E. esula* compared with grass hay. Similar positive associative effects have been reported for other high-protein supplements and forage species (e.g., Chase and Hibberd, 1987; Cassida et al., 1994). Conversely, adding *E. esula* stems to grass hay decreased IVDMD, which may reflect the lower CP and higher NDF and CT content of *E. esula* stems compared with grass hay.

Similarly, there were positive associative effects on microbial gas production when *E. esula* leaves, flowers, and stems from June and July were added to grass hay, up to a point. Microbial gas production was highest for the 75 : 25 mixtures of material collected in June and July indicating that adding *E. esula* to grass hay would stimulate rumen microbial activity in ruminants until 75% of their total diet in early to mid-summer is *E. esula.* For sheep, this percentage is greater than results from a one-week feeding trial where intake declined when the percent of *E. esula* hay exceeded 60% (Walker et al., 1994b). The response was similar whether the other hay in the mix was high-quality alfalfa or low-quality smooth brome, indicating that secondary compounds in *E. esula*, not forage digestibility, were limiting intake. In the field, intake of *E. esula* by sheep seldom exceeds 50% of the diet (Landgraf et al., 1984; Bartz et al., 1985; Olson et al., 1996). This may simply reflect that animals prefer variety when given a choice (Provenza, 1996) and not necessarily reflect the secondary chemistry of *E. esula.*

From material collected in August, microbial gas production increased with increasing quantities of leaves and flowers added to grass hay, but decreased with increasing levels of stems added. Presumably, this reflects the higher CP content

of leaves and flowers of *E. esula* compared with grass hay and the higher CT and NDF of stems compared with grass hay.

Usually 100% *E. esula* diets were associated with the lowest microbial activity but highest microbial purine concentrations. Low microbial activity at 100% *E. esula* may reflect that only microbes adapted to secondary compounds in *E. esula* are active at this level. The sheep that donated rumen microorganisms for this study had not been exposed to *E. esula* and thus the rumen microbial populations did not have time to adjust to this plant. Changes in diet highly influence numbers and relative proportions of different microbial species in the rumen (Yokoyama and Johnson, 1988). Several days to several weeks may be needed for microbial populations to adjust fully to new diets.

Usually microbial masses from the 100:0 *E. esula*–grass hay mixtures of leaves and flowers were much higher than the mixtures containing grass hay. This may reflect the fact microbial species that tolerate or thrive on *E. esula* increase rapidly when *E. esula* is added to the diet or that dead microorganisms accumulate when high levels of *E. esula* are in the diet. The technique we used cannot differentiate live from dead microorganisms.

CONCLUSIONS

Relatively high levels of *E. esula* leaves and flowers did not adversely affect sheep rumen microbial activity. Early in the growing season, leaves from previously defoliated *E. esula* plants did not negatively affect sheep rumen microbial activity, indicating that defoliation did not induce a strong defense response or that the overall high nutritive value of *E. esula* offset any negative effects associated with secondary compounds. In contrast, stems collected in July and August tended to have a negative effect on rumen microbial activity and mass, which correlated with seasonal increases in CT concentrations in stems. This may partly explain why sheep avoid *E. esula* stems when foraging, especially late in the growing season.

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CHEMOSENSORY ORIENTATION OF THE ROCK CRAB *Cancer irroratus*

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Abstract—The importance of chemical cues in the foraging behavior of the rock crab, *Cancer irroratus*, was investigated. Crabs were presented with mussel prey located upstream, downstream, or cross-stream. Trials were conducted under both light and dark conditions. With the prey upstream, the crabs exhibited the shortest search time and 100% searching success. There was a significant difference between flow direction treatments, but there were no significant differences between light and dark treatments. When presented with mussel extract and a seawater control, crabs approached the source of the current. Analysis of search patterns revealed differences in search time, path length and straightness, and total number of turns. Chemoreception was the predominant mode of prey detection and was used in guiding crabs to their prey. Large variabilities in the search path and search path parameters were exhibited, and there was no fixed search pattern for orientation toward food while foraging. It is suggested that rock crabs employed both chemotaxis and rheotaxis for locating odor sources.

Key Words—Crustacea, crabs, foraging, chemosensory orientation, chemoreception, odor, vision, path analysis, track analysis.

INTRODUCTION

Rock crabs, *Cancer irroratus* Say 1817 (Crustacea: Brachyura), spend most of the year at depths up to 700 m, on sandy or rocky substrates (Shotton, 1973; Haefner, 1976), where there are no visual cues available for food detection.

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Therefore, chemosensory cues should be particularly important for a crab's orientation towards food while foraging.

Various animals use chemical cues for guidance to food sources, and it is believed that many species switch from chemical cues at long distances to visual cues at short distances (Bell, 1991). Distance chemoreception plays a major role in food finding in crustacean predators and scavengers that are attracted by prey body odors and by the release of tissue fluids (Rittschoff, 1992; Rittschof and Sutherland, 1986). The lobster, *Homarus americanus*, uses chemical signals to determine source direction and distance of prey (Moore et al., 1991). Lobsters (*Panulirus interuptus*) and crabs (*Cancer antennarius* and *C. irroratus*) are attracted by food-related chemical stimuli (Zimmer-Faust and Case, 1982; Rebach, 1996).

Antennular mechanisms are used for distance chemoreception in rock crabs (Rebach et al., 1990), which are able to utilize low concentrations of chemical cues to detect, locate, and identity food items (Rebach, 1996). Ristvey (1993) demonstrated that rock crabs can distinguish the odor of blue mussel (*Mytilus edulis*) from that of the ribbed mussel (*Geukensia demissa*). There is some evidence that both chemical and visual cues may be used when close to the mussel (unpublished data). These data are not sufficient to understand the interaction between visual and chemosensory stimuli.

There are no reported studies on the search patterns of the rock crab. Quantification of the movement patterns of foraging crabs is necessary for understanding the way crabs discover prey items. Search orientation paths may reflect the functional design of a search pattern (Bell, 1991) and may be an important determinant of a predator's success in encountering prey items (Aluja et al., 1989).

Movement patterns and/or search paths of mollusks, insects, echinoderms, fish, reptiles, birds, and mammals have been quantified under both natural and artificial conditions. Many species have a tendency to move along a straight path, or alternate right and left turns to keep their overall course on a straight path (Smith, 1974a,b; Thomas, 1974; Jones, 1976; Bond, 1980; Mellgren and Roper, 1986; Dusenbery, 1989; Tye, 1989; Veena and Ganeshaiah, 1991). Dusbenbery (1990) proposed that searching animals could move accurately upwind or upstream within a chemical plume and move efficiently to the source. However, the walking speeds and heading angles of *Homarus americanus* changed with distance to odor source (Moore et al., 1991).

To determine whether chemosensory stimuli provide cues for source direction and distance in guiding foraging crabs to prey, we analyzed their ability to detect prey, search paths, and search behavior. Our first objective was to ascertain whether olfaction was the predominant sense used by rock crabs, in response to chemical and visual stimuli, to detect ribbed mussel prey. Our second objective was to describe and analyze the crabs' search path, quantify their two-dimensional movement patterns, and evaluate the mechanism of odor orientation.

METHODS AND MATERIALS

Experimental Animals. Intermolt rocks crabs of 71–130 mm carapace width were captured by commercial fishermen off the coasts of Maryland and Delaware. Crabs were maintained in running seawater for at least 10 days prior to testing. All crabs were labeled. Body size, weight, and leg condition were recorded. Only intact, undamaged crabs were used in the tests. Each crab was fed once daily with a single mussel during holding, but was deprived of food for two days before each trial. Ribbed mussels, *Geukensia demissa*, were collected by hand from salt-marshes in Girdletree, Maryland, and maintained in the laboratory.

Apparatus. All tests were conducted in a flow-through tank (187 × 93 × 62 cm) with a total capacity of 720 liters, using filtered, artificial seawater of 14–16°C and 30–35 parts per thousand (ppt) salinity. Illumination was provided by three 40-W fluorescent lights (day) or two 85-W infrared lights (night).

In experiment I, seawater entered one end of the experimental tank through 15 holes in the L-section of PVC pipe, flowed underneath a Plexiglas tank divider raised 5 mm from the bottom, and drained through an outlet at the other end. This produced an even flow across the bottom of the tank (Figure 1). Water flow rate and direction were controlled by valves, allowing experiments to be performed on either side of the tank. The carrier flow rate, determined by measuring outflow, was 5.7 ± 0.1 liters/min and the water flow velocity, determined by measuring the progress of a dye front across the tank, was 61.0 ± 0.1 cm/min.

In experiment II, the L-shaped pipe was replaced by an inflow pipe with one hole and the tank divider was removed (Figure 1). Dye tests indicated that test odors injected through the stimulus inlet reached the far end of the tank within 30 sec, and a fan-shaped odor plume extending from the mouth of the inflow pipe to the downstream end of the tank was established about 1 min after introduction of the stimulus.

An infrared-sensitive videocamera (RCA TC 1809) with a wide-angle lens was mounted 126.5 cm above the test tank. The camera was connected to a sequential switcher (PELCO), which was time-base controlled either directly (Hotronic AD 51) or through a VCR (Panasonic AG 1960). An image could be selected either from the camera or VCR, transferred to a Gould FD 5000 image processor (a programmable data acquisition and image display system) controlled by a DEC PDP 11/44 minicomputer, and then displayed on a video monitor (Mitsubishi HF 1400 K). This combination permitted recording of the animal's path and storing the information as a time series of x and y coordinates.

The minicomputer allowed us to track crab movements from the screen of the monitor in real time. Two cursors representing the initial positions of the crab and mussel were diplayed on screen. A trackball was used as the cursor control

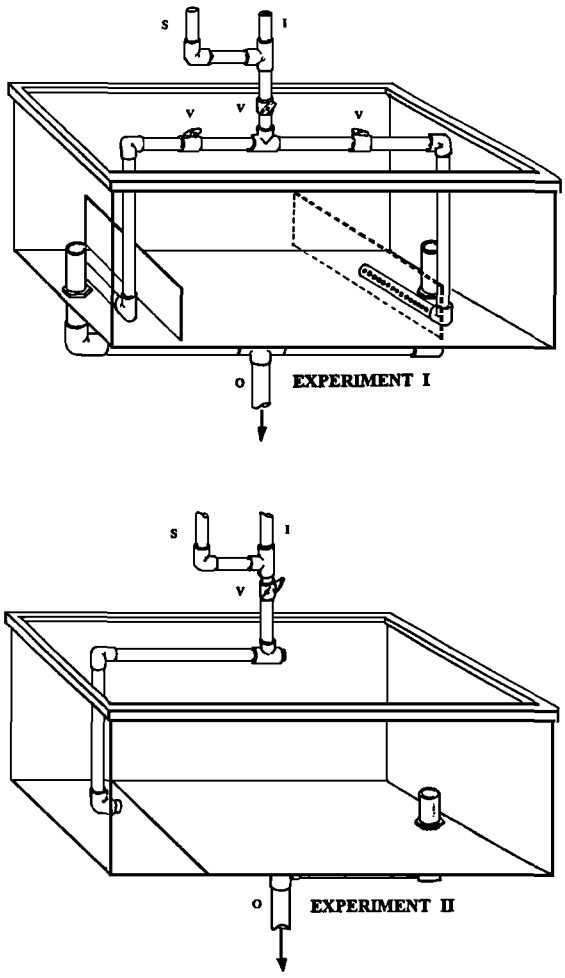

FIG. 1. Three-dimensional view of experimental tanks. Water flow direction in experiment I can be controlled by opening or closing valves. I = inflow pipe, O = outflow pipe, S = stimulus inlet pipe, V = valve.

device. The path of the crab was followed by moving its cursor by trackball, producing a continuous record of its path. Stops, turns, and other behaviors were recorded by depressing assigned keys on the terminal's keyboard during their occurrence.

Experiment I: Importance of Odor in Searching Success. To determine the importance of odor in foraging, water flow over prey items was presented in

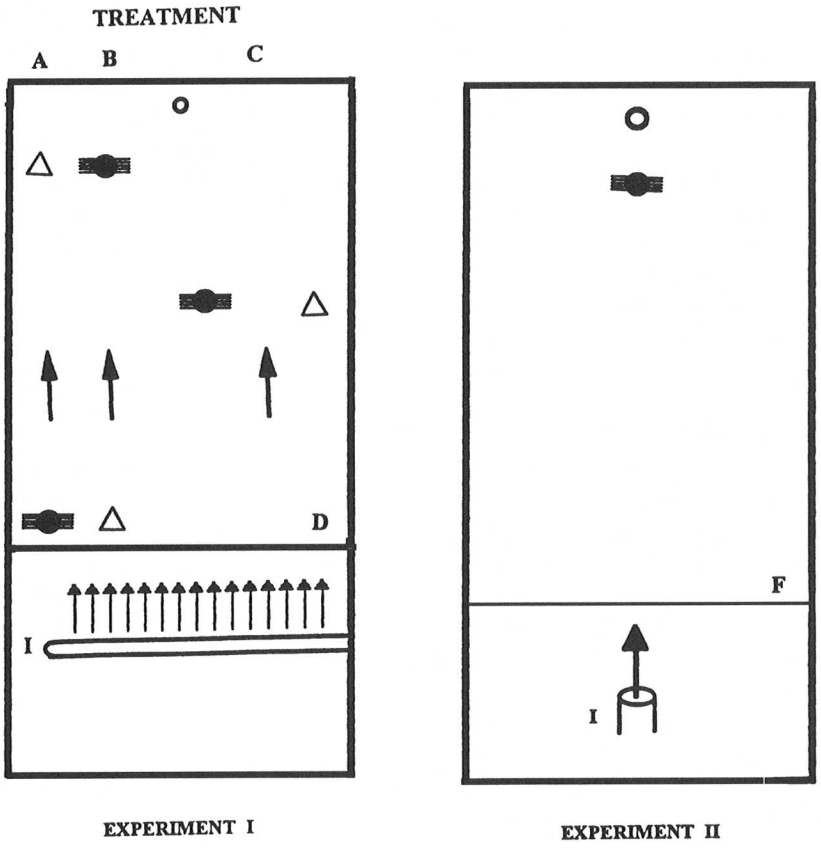

FIG. 2. Diagram of experimental design and water delivery system, showing water flow direction and relative position of mussel and crab in experiment I and crab position and odor stimulus input for experiment II. ⟶ = water flow direction, △ = mussel, ▬● = crab, I = inflow pipe, O = outflow pipe, D = tank divider, F = finish line, A = prey downstream treatment, B = prey upstream treatment, C = prey cross-stream treatment.

three directions (Figure 2): (1) prey upstream treatment—water flowed over the mussel first and the water current carried mussel odor toward the crab; (2) prey downstream treatment—water flowed across the crab first and mussel odor was not carried toward the crab; and (3) prey cross-stream treatment—water passed over both the mussel and the crab at the same time and, therefore, no mussel odor was carried toward the crab by the current.

Tests were conducted between 09:00 and 18:00 hr. For each treatment, a total of 15 crabs was observed in the light. Crabs were randomly chosen and used

only once. Each test was conducted by covering a crab with a perforated plastic cup on one side of the tank, and presenting a live, but cracked, mussel of 52 ± 2 mm length (measured as the greatest anterior-to-posterior distance) at the other side of the tank, opposite the crab. Tank side was randomly chosen to avoid side and light biases. The crab was released and its path tracked and videotaped for 1 hr. A trial was considered as a successful search only if the crab attempted to open the mussel. A trial was terminated if a crab did not move within 60 min of release and those data were not used. Each treatment was repeated in the dark under infrared light.

Experiment II: Search Path Analysis. Fifteen crabs were tested in the dark between 09:00 and 18:00 hr with a chemical stimulus and a seawater control. There was at least a 1-hr interval between tests to ensure that no odor remained from the previous test. Crabs were placed at the downstream end of the tank and given time for their initial agitation to decrease. If this did not occur within 20 min, the crab was replaced. Mussel extract, freshly prepared before testing, was used as a stimulus and introduced into the stimulus inlet pipe once the crab was settled. The extract was prepared by cracking the shells of live mussels and draining body fluid. Fifty grams of body fluid was dissolved in 10 liters of seawater to produce a concentration of 5 g/liter. Control tests were performed with seawater substituted for the mussel extract. Each crab treatment also served as its own control to reduce error due to individual differences. A test was terminated when the crab crossed the upstream finish line (Figure 2) or 5 min after stimulus introduction, whichever came first. A trial was considered unsuccessful if the crab did not cross the line in 5 min or only walked along the tank wall.

Data Analysis. In experiment I, we recorded successful search times of individual crabs and time spent foraging until finding the mussel. The percentage of crabs locating a mussel was calculated by dividing the number of successful trials by the total number of trials.

The search path analysis program generated summaries of: (1) x and y coordinates; (2) relative speed of each individual crab; (3) search time: time from first movement to cross the upstream finish line; (4) individual path lengths (individual displacements): distance between two recording points; (5) total path length (gross displacement): sum of all individual displacements; (6) path straightness: the straight-line distance (distance between release point and mussel) divided by total path length; (7) locomotory rate (average speed of movement): total path length divided by time; (8) net displacement: distance between the release point and the end point; and (9) distance to goal: displacement of the crab from the mussel at a given time.

Other variables used to describe search patterns included: percentage of crabs locating a mussel, percentage of time spent moving (time spent moving as a fraction of the total search time), percentage of time spent resting (time spent resting as a fraction of the total search time), search latency (time interval

between the stimulus introduction and first movement of the crab), total number of turns, and total number of stops.

To correct nonnormality and heterogeneity of variance, data were log-transformed and calculated percentages were arcsin-transformed before statistical testing (Zar, 1984). However, actual means and standard deviations are presented in the text. Differences in successful search times among the three treatments (upstream, downstream, cross-stream) under light and dark conditions were tested by two-factor ANOVA followed by a Tukey multiple comparison test. Differences between control and treatment parameters in experiment II were tested by a pairwise t test on transformed data, differences in variances by an F test on transformed data. A chi-square test was used to determine the path straightness and the extent to which a crab tended to travel in a straight line. Data were analyzed with SAS/STAT 6.11 (SAS Institute Inc., Cary, North Carolina).

RESULTS

Experiment I: Importance of Odor in Searching Success. Twenty trials were eliminated because of the nonmovement of crabs. The eliminated trials were all from prey downstream and cross-stream treatments: six from downstream, light; seven from downstream, dark; five from cross-stream, light; and two from cross-stream, dark. Lack of movement was related to current direction (Fisher's exact test, $P < 0.001$).

All crabs in the prey upstream treatment responded promptly and walked in a relatively direct path to the mussel, often along the tank wall. Most crabs in the downstream and cross-stream treatments walked back and forth along the tank wall or "rested." Crabs in all treatments made turns or stops while searching, often stopped at tank corners, and seldomly walked across the middle of the tank. They moved rapidly and grabbed mussels immediately after making the last stop before reaching the mussels, held the mussels with one claw, and removed the meat with the other claw.

Crabs in the prey upstream treatment (both light dark) exhibited the lowest average mean search times (3.17 and 2.15 min, respectively) and highest percentage of searching success (100%); downstream and cross-stream treatments had longer mean search times (11.55 and 11.57 min for light, 15.62 and 16.01 min for dark, respectively) and a lower success rate of finding prey (about 68%) (Figures 3 and 4). Search times ranged from 0.75 to 33.08 min for downstream and cross-stream treatments and the means exhibited large standard deviations (Table 1).

Table 1 summarizes ANOVA results for crab search time and percent success and for the treatments of flow direction and light condition. There was no significant difference in search time or percent success between light and dark treatments ($P = 0.98$ and 0.42, respectively), but there was a significant difference

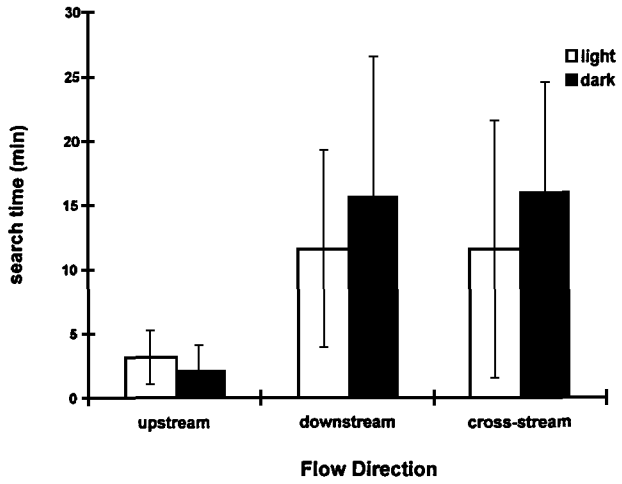

FIG. 3. Search time by flow direction and light conditions. Error bars indicate ±1 SD.

between treatment by flow direction ($P < 0.001$ for search time, $P = 0.003$ for percent success). For both light conditions, the search time for prey upstream was significantly lower than for downstream and cross-stream treatments, but there was no difference between downstream and cross-stream treatments (Tukey, $P = 0.05$) and there were no significant effects of the interaction between light and flow direction ($P = 0.06$).

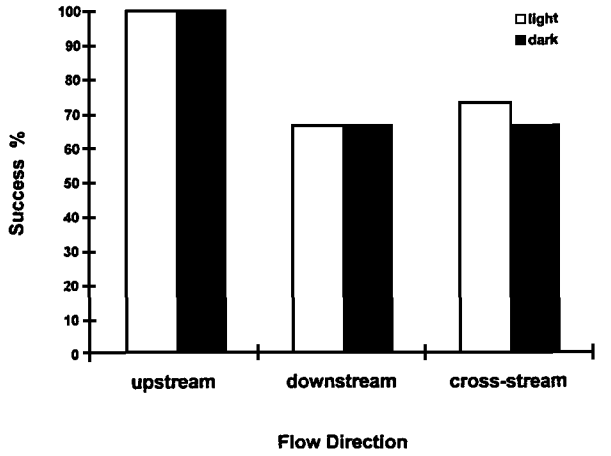

FIG 4. Searching success by flow direction and light conditions.

TABLE 1. SUMMARY OF RESULTS AND TWO-FACTOR ANOVA ON TRANSFORMED DATA FOR CRAB SEARCH TIME BY FLOW DIRECTION AND LIGHT CONDITION (LIVE MUSSELS—EXPERIMENT I)[a]

	Light		Dark	
	Search time (min) (Mean ± SD)	Success (%)	Search time (min) (mean ± SD)	Success (%)
Upstream	3.17 ± 2.09a	100	2.15 ± 1.93a	100
Downstream	11.55 ± 7.69b	66.7	15.62 ± 10.87b	66.7
Cross-stream	11.57 ± 10.05b	73.3	16.01 ± 8.53b	66.7
	Search time (min) (F, $P > F$)		Success (%) (F, $P > F$)	
Light difference	0.001	0.9756	1.00	0.4226
Current difference	26.96	0.001***	312.85	0.0032**
Light and current interaction	2.86	0.0645		

[a] P is the two-tailed significance probability of a greater F value; ** indicates significance at $P = 0.01$, and *** $P \leq 0.001$. Under a given light condition, treatments followed by different letters are different at $P = 0.05$ (Tukey's).

Experiment II: Search Path Analysis. When presented with an odor concentration gradient, crabs moved more rapidly than when presented with a single live mussel (Tables 1 and 2). Crabs seldom walked along the tank wall as they did in mussel tests. Many control group trials were eliminated because crabs failed to cross the finish line within 5 min while crabs in mussel extract treatments readily walked to the mouth of the tube where the mussel extract was released and exhibited feeding behaviors.

Individual crab tracks were never identical (Figure 5). Table 2 summarizes results and analyses for search path variables in control and mussel extract treatments. All variables except net displacement (initial distance between crab and odor source), which was approximately the same in all trials, had large standard deviations. A significant difference in variance occurred only in total search path length, although large variances were also observed in search time and search latency time.

The mussel extract treatment group had a significantly greater mean search time (127.39 vs. 69.71 sec), longer search path lengths (476.66 vs. 230.23 cm), and a greater number of turns (34.07 vs. 13.78) than the control group. The treatment group made more turns at the beginning stages of searching than at the end, exhibited increased search path widths, and did not walk in straight lines. The control group exhibited straighter paths than the treatment group (0.63 vs. 0.37). If a crab walked in a straight line, the expected value for path straightness would be 1.0. A chi-square test showed no significant difference from 1.0 for the

TABLE 2. SUMMARY OF RESULTS AND ANALYSIS OF SEARCH PATH VARIABLES (MUSSEL EXTRACT, EXPERIMENT II)[a]

Behavioral variables	Response (mean ± SD)		t	$P > t$	F	$P > F$
	Control	Treatment				
Search latency time (sec)	63.97 ± 104.87	66.77 ± 53.43	1.66	0.1211	2.90	0.0652
Search time (sec)	69.71 ± 51.94	127.39 ± 58.27	3.32	0.0055**	3.05	0.0541
Total turns (N)	13.78 ± 7.85	34.07 ± 22.47	3.33	0.0055**	1.22	0.7304
Total stops (N)	1.71 ± 1.73	1.57 ± 1.09	0.33	0.7474	2.28	0.1507
Total path length (cm)	230.23 ± 65.72	476.66 ± 251.65	4.17	0.0011**	3.28	0.0410*
Net displacement	135.48 ± 8.37	135.85 ± 10.22	0.10	0.9215	1.52	0.4577
Path straightness	0.63 ± 0.19	0.37 ± 0.19	4.10	0.0013**	1.40	0.5530
Movement speed (average, cm/sec)	6.19 ± 3.92	4.24 ± 2.53	1.75	0.1041	1.53	0.4527
Time spent moving (%)	79.72 ± 24.55	83.39 ± 17.17	0.06	0.9504	1.71	0.3476
Time spent resting (%)	20.28 ± 24.55	16.61 ± 17.17	0.46	0.6523	2.24	0.1598

[a] P is the two-tailed probability of a greater t or F value. Means were tested with a paired t test on transformed data, variances with an F test on transformed data. *Indicates significance at $P = 0.05$, **$P = 0.01$. Control $N = 14$, Treatment $N = 14$.

control group ($\chi^2 = 2.3393$, $P > 0.05$) suggesting a straight path, while there was a significant difference for the treatment group ($\chi^2 = 6.707$, $0.01 > P > 0.001$), indicating that this group did not travel in a straight line.

DISCUSSION

Crabs detected upstream prey items more often and more quickly than downstream and cross-stream prey. Because there was no difference in search times or success rates between downstream and cross-stream treatments, we can exclude the possibility that current direction significantly affected the movements of crabs in those tests. Crabs in the prey upstream treatment did not move across or down the current. Finding prey items in downstream and cross-stream treatments may have been due to random searching; successful search times among individuals were highly variable. Mussels do provide both chemical and visual cues for orientation. Crabs in the prey downstream and cross-stream treatments required more time (but not significantly more) to find mussels in the dark than in the light. However, these crabs eventually did locate the prey items. The lack of difference in search times or success rates between light and dark trials suggests that visual cues were less important than chemical cues. The increased directness of path as the crab approached the mussel may be due to the use of both chemical and visual cues as crabs neared prey items. However, chemoreception was the predominant sensory modality used by rock crabs to detect prey, and

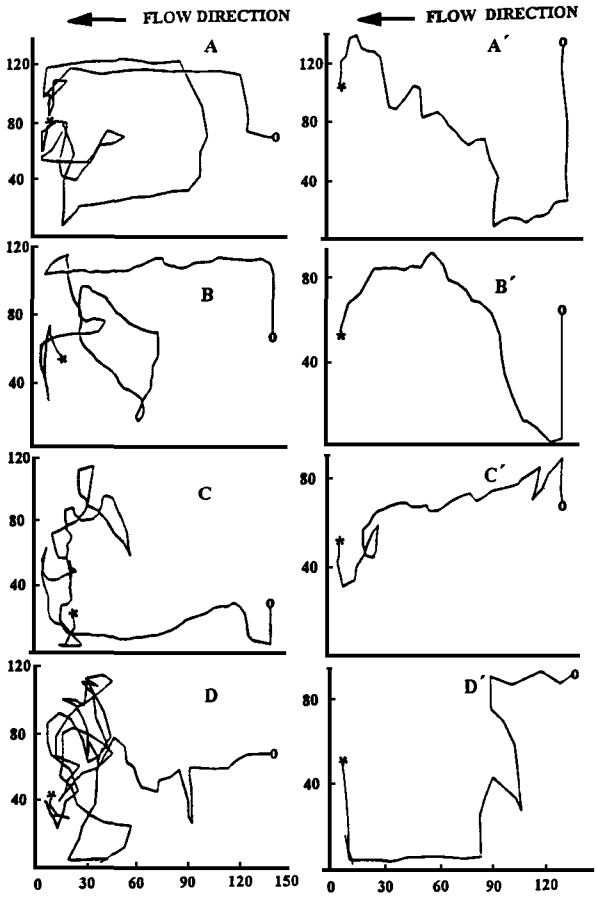

FIG. 5. Representative search paths from mussel odor treatments (A–D) and control (A′–D′). Each crab was used once for both the treatment and control trial. The x and y axes represent two sides of the tank and a series of x and y coordinates gave rise to the search path. Water flows from right to left. O designates the odor source, an asterisk designates the starting point of the crab.

chemical cues played an important role in guiding the crab to that prey. Of the 20 crab trials that were eliminated because the crab failed to move, all involved currents that did not reach the crab because the odor source was downstream (13) or cross-stream (7) (11 were in the light, nine were in the dark).

Based on the large variability in search paths and the large standard deviations of path attributes, there does not appear to be a fixed search pattern for the crabs' orientation toward food. The results are similar to those observed in

the lobster, *Homarus americanus* (Moore et al., 1991), in which there was large variability in search paths and high standard deviations in measured behavioral variables in multiple trials of the same individual and among members of the test population. Lobsters also have been observed to move more slowly when tracking odors (Atema, 1996).

Searching strategies differed between the whole mussel and mussel extract studies. When presented with whole mussels, crabs walked along the tank wall instead of walking in the open area, whereas crabs seldom walked along the wall when presented with mussel extract. This may be related either to the nature or the concentration of odor in the plume. The concentration of mussel tissue fluid used in the path analysis study was greater than the concentration of whole mussel body odor in the current direction study. Chemical cues released from whole prey (body odor) provide stimuli for investigative and attractive behaviors, while prey tissue extracts also provide stimuli for feeding behaviors (Zimmer-Faust, 1993). Average search times exhibited by crabs in the prey upstream (live mussel) and path analysis (mussel extract) treatments were the same (2.15 vs. 2.12 min). However, control times in these two experiments were very different. Crabs exposed to the seawater control in the extract experiments took 64 sec to reach the current source, while crabs in prey downstream and cross-stream situations, lacking both odor- and current-borne information, took between 11 and 16 min. This emphasizes the importance of a downstream current in the acquisition and utilization of chemosensory information. Further study might determine the relationship between concentration of the odor source, current strength and direction, and searching strategy used.

The acquisition of odor cues in the extract experiments initiated a more deliberate searching strategy since the control group had shorter search times, shorter and straighter search paths, and fewer turns than the extract group. Search patterns were not uniform in the presence of prey odor and differed at different stages of the search. The paths exhibited decreasing turn frequency and search width, thereby increasing their directedness, only as the crab neared (approximately 10 cm) the odor source. Differences in search times between extract and control groups were statistically significant despite large variances. The large variances in search latency time also indicate the variability of individual responses to the odor source. Average speed of movement, total number of stops, and percentage of time moving or resting were not affected by the presence of chemical cues. Unlike flying insects (Aluja et al., 1989; Tourtellot et al., 1991), which traveled less distance, spent less time, and flew with an increased directness to a model with odor than a model without odor, walking rock crabs responded to odor by increasing search path length and decreasing directness. They followed a more convoluted, nonlinear path, similar to that observed in lobsters (Moore et al., 1991; Atema, 1996), whereas the control group exhibited a straight locomotory pattern. This behavior fits Jander's (1975) hypothesis that

when environmental cues are not available, straight movement is the optimal, or most efficient, strategy for patchy resources.

Because our odor stimuli were delivered from a point source, resulting in a narrow detection zone, a straight path would be a poor strategy for locating a small source (Jander, 1975), and odor-treatment crabs did not walk in a straight line. The straight-line movement of the control group (no odor cues) did not differ greatly among individuals, but the paths used to reach the odor source were more variable. Although flying insects do move accurately up-current in an odor plume (Dusenbery, 1990; Bell, 1991), our results suggest that some decapods may not move uniformly upstream once within the influence of the plume.

Animals may use different mechanisms to orient toward food. The large variability in crab search paths may indicate variation or noise at the level of the central nervous system. Our results suggest that there was no internally stored orientation information or motor program that determined crab search patterns. The water current provided two possible external signals for distance orientation: a directional odor gradient and a velocity gradient. There are therefore two orientation mechanisms that could be used to localize the source; chemotaxis—comparing chemical intensities either temporally (klinotaxis—typically resulting in a zig-zag search path) or spatially (tropotaxis), and rheotaxis—using velocity gradients in water.

In this study, it is likely that chemotaxis and rheotaxis were both employed. Based on search times of control groups in both experiments, the presence and direction of current influenced the speed and the strategy of search. In control trials of the whole mussel studies, in which the current did not deliver an odor directly to the crab, prey was not easily found (or the crab did not move). Up-current movement occurred in both experiments, whether or not there was an odor. If an odor was present, crabs did not exhibit a direct up-current path to the odor source (as they did when odor was not present). The search paths of rock crabs were straight, zig-zag, or convoluted at different segments. To further understand the research mechanisms, it may be necessary to analyze changes in turning angle as well as locomotory rate along the search path. It would also be useful to further examine distance to goal, reactive distance and path straightness as mean vectors at each recording point. This would provide more information on search intensity.

The American lobster, *Homarus americanus* (Moore et al., 1991), and Florida spiny lobster, *Panulirus argus* (Reeder and Ache, 1980), use both klinotaxis and tropotaxis to locate an odor souce. When only chemical information is available, an insect can switch between tropotaxis and klinotaxis for odor source localization, depending on the slope of the chemical gradient (Bell and Cardé, 1984), but can also use rheotaxis in an odor plume (Dusenbery, 1990). Devine and Atema (1982) suggested that chemically stimulated rheotaxis, in which predators are stimulated by prey odor to search with mechanoreceptors, might provide a useful redundancy of sensory input. Chemical senses are closely

allied with mechanoreceptors both morphologically and functionally. Crustacea have numerous chemoreceptors and mechanoreceptors located on antennules, mouthparts, chelae, and walking legs (Derby and Atema, 1982; McLaughlin, 1982). Rock crabs may employ these receptors in odor containing bottom currents for prey detection and localization.

In conclusion, chemoreceptor was the predominant sense used in the detection of prey odors, and chemical cues played an important role in guiding rock crabs to prey items. There was no fixed search pattern for orientation toward food while foraging. It is suggested that rock crabs employed both chemotaxis and rheotaxis for locating odor sources. In the absence of odor and the presence of a downstream current, the strategy appeared to be "move upstream, investigate whether there are odors present, then switch to a search pattern." In deep water, rock crabs may detect prey through a search pattern of moving into a current, and then turning, looping and zig-zagging in the presence of chemosensory information.

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FRUGIVORY AND TASTE RESPONSES TO FRUCTOSE AND TANNIC ACID IN A PROSIMIAN PRIMATE AND A DIDELPHID MARSUPIAL

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Abstract—The taste responses to sweet and astringent compounds were investigated in two mammals of similar ecology, by using the classical method of the two-bottle test. The taste threshold for fructose was higher in *Microcebus murinus*, a prosimian primate, than in *Caluromys philander*, a didelphid marsupial. The profiles of suprathreshold responses resembled a dissymmetric bell-shaped curve, but the rate of consumption of sweet solutions up to maximal intake increased more rapidly in *Microcebus* than in *Caluromys*. Despite showing a photoperiod-synchronized physiology, *Microcebus* displayed no seasonal variation of the taste threshold and suprathreshold responses. The depressing effect of tanning acid on the ingestion of fructose solutions increased progressively with tannin concentration and was lower as fructose concentration increased. Inhibition thresholds for tannic acid were similar between the two species. The data suggest that adaptation to frugivorous diets is associated with globally similar shaping of the taste responses, even though subtle differences of palatability may account for differences of feeding selectivity.

Key Words—Discrimination, sugar, tannin, ecological convergence, taste threshold, seasonality, primate, Marsupialia.

INTRODUCTION

Taste thresholds, mainly for sucrose have been recorded in a growing number of mammals in an attempt to clarify the role of taste perception in the differentiation of feeding behavior (Glaser, 1986; Simmen and Hladik, 1998; Ramirez,

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1990). In a recent paper reviewing taste thresholds for soluble sugars across a wide range of primate species (Simmen and Hladik, 1998), the question was raised whether phylogenetic relatedness rather than adaptation to similar biochemical characteristics of diet accounted for the similarity of gustatory abilities within a few groups of species. To define whether the use of equivalent food sources corresponds to convergent shaping of the taste responses toward alimentary stimuli, we conducted a study in which we compared the taste responses of two tropical mammals showing ecological convergence and belonging to very distantly related taxa—the lesser mouse lemur (*Microcebus murinus*, Primates: Cheirogaleidae) and the bare-tailed woolly opossum (*Caluromys philander*, Marsupialia: Didelphidae).

Among prosimian primates, the Malagasy cheirogalids, like their African counterparts, the lorisids, exhibit ecological convergences with American didelphid marsupials (Charles-Dominique, 1983). These include nocturnality, arboreal habits, small to medium body sizes, and diets based on the pulp of ripe fruits, floral nectars, and invertebrates, mainly insects. The two species studied here belong to taxonomic groups that have retained ancestral features but are not strictly equivalent in that *Caluromys philander* is larger than *Microcebus murinus*.

In a first series of tests, taste thresholds as well as the profiles of suprathreshold responses to fructose solutions were compared between the two species. Data on suprathreshold responses are poorly documented, although they provide insights into the pleasantness of stimuli. This sensory dimension is assumed to be of major importance in food selection of many species coping with highly diversified environments such as tropical forests. In addition, we examined whether the lesser mouse lemur displayed a seasonal variation in its taste responses toward fructose. Indeed, this pseudohibernating species undergoes a body weight change of about 30% between the fattening and the leaning period of its annual cycle (Petter-Rousseaux, 1974), with corresponding variations of the taste threshold for sucrose under certain experimental conditions (Simmen and Hladik, 1988).

In a second series of tests, the responses of the two species toward mixtures of fructose and tannic acid were compared. The widespread occurrence of tannins in plants (Swain, 1979) and their commonly assumed biological significance (i.e., to prevent consumers from feeding) (Feeny, 1970; Lebreton, 1982; Robbins et al., 1987) make the analysis of their effect on taste of particular interest. There is experimental evidence that "astringency" corresponds to one or several taste qualities. In rodents, the chorda tympani nerve as well as isolated cells in the gustatory cortex respond to tannic acid (Kawamura et al., 1969; Schiffman et al., 1992; Kosar and Schwartz, 1990). Recently, a gallotannin has been shown to elicit a signal on the chorda tympani proper nerve—which only conveys gustatory signals—of *Microcebus murinus* (Hellekant et al., 1993a) and to produce large responses in a neuronal population of the macaque orbitofrontal cortex

(Critchley and Rolls, 1996). A few studies in the field of chemical ecology provide empirical evidence that primate species vary in their tolerance of condensed tannins (Ganzhorn, 1988; Barton et al., 1993). It is, however, not experimentally demonstrated to what extent such a trait varies among fruit-eaters and whether the perception of "astringency" is correlated with body size, as found for soluble sugars in primates (Simmen and Hladik, 1998). An allometric relationship might be expected since natural food intake increases with body weight across species [e.g., in primates (Barton, 1992)], and the efficiency of a tannin in reducing protein digestibility is concentration-dependent.

In this study, a rejection profile was determined and the inhibition thresholds for tannic acid were compared with electrophysiological data recorded in the lesser mouse lemur (Hellekant et al., 1993a).

METHODS AND MATERIALS

Rearing Conditions. Study animals were all sexually mature young adults and adults. They were housed in individual cages (160 cm long × 75 cm wide × 250 cm high) under constant temperature (24°C), and were exposed to an artificial photoperiodic schedule. For *Caluromys philander*, daylength was maintained constant throughout the year (12L : 12D), as occurs in the equatorial habitat. The artificial light cycle applied on *M. murinus* lasted only eight months (see Perret and Schilling, 1987), including five months of long daylength (14L : 10D) and three months of short daylength (8L : 16D). It is well established that daylength variations synchronize the seasonal changes in the physiological and behavioral activities of the lesser mouse lemur (Petter-Rousseaux, 1974; Perret, 1979). Fattening, subsequent torpor, and decrease in metabolic rate occur during the period of short daylength. Animals were fed on standard animal food (monkey biscuit) mixed with cereal and milk, supplemented with a variety of fruits and fresh water. Body weights were recorded before and at the end of the experiments.

In the first experiment with fructose, nine male *M. murinus* 7 months to 2 years old, and 16 *C. philander*, 3–6 years old, including eight males and eight nonpregnant females, were used. In the second experiment, the same *C. philander* individuals were tested but we could test only eight different male *M. murinus* 1–3 years old. The consistency of their pattern of ingestion of fructose solutions versus water with that of the animals studied in the first experiment was checked.

Test Procedure and Statistical Analysis for Comparison of Taste Thresholds and Suprathreshold Profiles. In the first experiment, we determined taste thresholds for fructose, and we established the profiles of ingestive responses to suprathreshold sugar concentrations with the two-bottle test method. The procedure (Simmen and Hladik, 1988), derived from that of Glaser (1979), can be sum-

marized as follows: the position of the two feeding bottles (tap water versus fructose solution) as well as the concentrations of the fructose solution—20 concentrations ranging from 27 mM to 2 M in *Microcebus* and 32 concentrations ranging from 1.6 mM to 1.03 M in *Caluromys*—were varied at random during each trial. Tests started after completing a period of habituation, during which the individuals were supplied with a high concentration of sugar solution and tap water simultaneously. Habituation was considered achieved when a marked preference for the fructose solution was recorded over four successive trials. Initially, the bottles were left during the whole activity period and were progressively made available in ad libitum amounts for a short period lasting 3 hr in *Microcebus* and 30 min in *Caluromys*. The difference in duration is justified by the differential fluid intake observed between a primate species weighing approximately 80 g and a marsupial weighing about 400 g. Tests were performed before the animals were fed their daily meal. We recorded the intakes of sugar solutions and tap water and clustered the results in successive concentration intervals. The threshold is defined as the lowest concentration interval (with the minimum width) for which the mean difference of consumption between the sugar solution and tap water is significant ($P \leq 0.05$ or lower with a paired-sample t test). Lesser mouse lemurs were tested during each of the two distinct phases of the photoperiodic schedule to assess the possibility of a seasonal variation of taste responses.

Following this procedure, subjects were tested with slightly different ranges of concentrations. Each series of experiments ended at a strictly determined date corresponding in *Microcebus* to the change in the photoperiodic schedule (long daylength versus short daylength), but the onset of the experiment for each individual depended on the time required to reach the criterion for full habituation. Similarly, in *Caluromys*, all animals were tested within scheduled dates to avoid the possibility of a seasonal shift in the responses. Accordingly, when clustering the results, responses of individuals that could not be tested with each of the concentrations within a given interval were discarded. This allowed individual animals to enter equally into the calculation of P for each block of concentrations.

The profiles of suprathreshold responses were determined by plotting the amount of fructose solution ingested against sugar concentration. The resulting profiles resembled a dissymmetric bell-shaped curve. In order to facilitate statistical treatment of the results, especially comparison between the two phases of the fattening cycle in individual lesser mouse lemurs and differences between the two species, we transformed the data into decimal logarithms and applied linear regression models separately on the increasing and decreasing parts of the profile, allowing for an analysis of covariance of resulting slopes.

Test Procedure to Measure Effects of Tannic Acid on Ingestion of Fructose Solutions. In a second experiment, we recorded the effect on ingestion of adding various tannic acid concentrations ($C_{76}H_{52}O_{46}$, MW: 1701.23; Fluka) to

fructose solutions. Commercially available gallotannin includes various forms of galloyl-glucose (Dr. A. E. Hagerman, personal communication), which are expensive to purify in large quantities. However, unpurified tannins have been shown to reduce weight gain effectively in voles (Lindroth and Batzli, 1984). To minimize bias due to variation in the chemical composition that may arise from independent tannin extractions, we used tannic acid from the same batch as that utilized on *Microcebus* by Hellekant et al. (1993a) and on rhesus macaque by Critchley and Rolls (1996). The sugar concentrations range from those close to the threshold of each species to those eliciting large consumption (100 and 300 mM in *Microcebus* and 30, 150, and 300 mM in *Caluromys*), as revealed in the first tests. Tannin concentrations ranged between 0.11 mM and 1.27 mM in *Microcebus* and between 0.03 mM and 7.62 mM in *Caluromys*. Each mixture of tannin and sugar was presented simultaneously with tap water in a two-bottle test, with tannin concentration varied at random. The amounts ingested were recorded and compared with those obtained separately (to prevent the animals from overfeeding) for corresponding pure fructose solutions. The efficiency of the deterrent effect is measured in terms of inhibition threshold; that is, the concentration of tannin from which the mean difference of ingestion between the mixture and the fructose solution is significant ($P \leq 0.05$ with a paired-sample t test), and in terms of percentages of inhibition of the consumption.

RESULTS

Taste Thresholds for Fructose. As expressed in Figure 1, the taste threshold for fructose of lesser mouse lemur males is 44–51 mM in both the long daylength phase and the short daylength phase of the light cycle ($P < 0.05$ and $P < 0.02$, respectively, with $N = 18$ in each case). The threshold of male woolly opossums is 2.5–5 mM: $P < 0.02$, $N = 14$; Figure 1), that of female woolly opossums 20–25 mM ($P < 0.01$, $N = 8$).

Suprathreshold Responses to Fructose. Figure 2 shows the mean intake of fructose solution per unit body weight of mouse lemurs and of woolly opossums plotted against the logarithm of sugar concentration. The profiles resemble a dissymmetric bell-shaped curve with maximal intake of the fructose solutions obtained for a concentration of about 450 mM in *Microcebus* and 350 mM in *Caluromys*.

Following logarithmic transformation of both axes, the regression lines for the two distinct parts of the profile (slopes A and slopes B in Table 1), were calculated as follows: for each animal, slope A included all data between the threshold and the concentration above which the tendency to ingest large amounts of sugar solutions was inverted. To avoid arbitrary definition of this upper boundary, an iterative method was applied in which r^2 was calculated step by step for

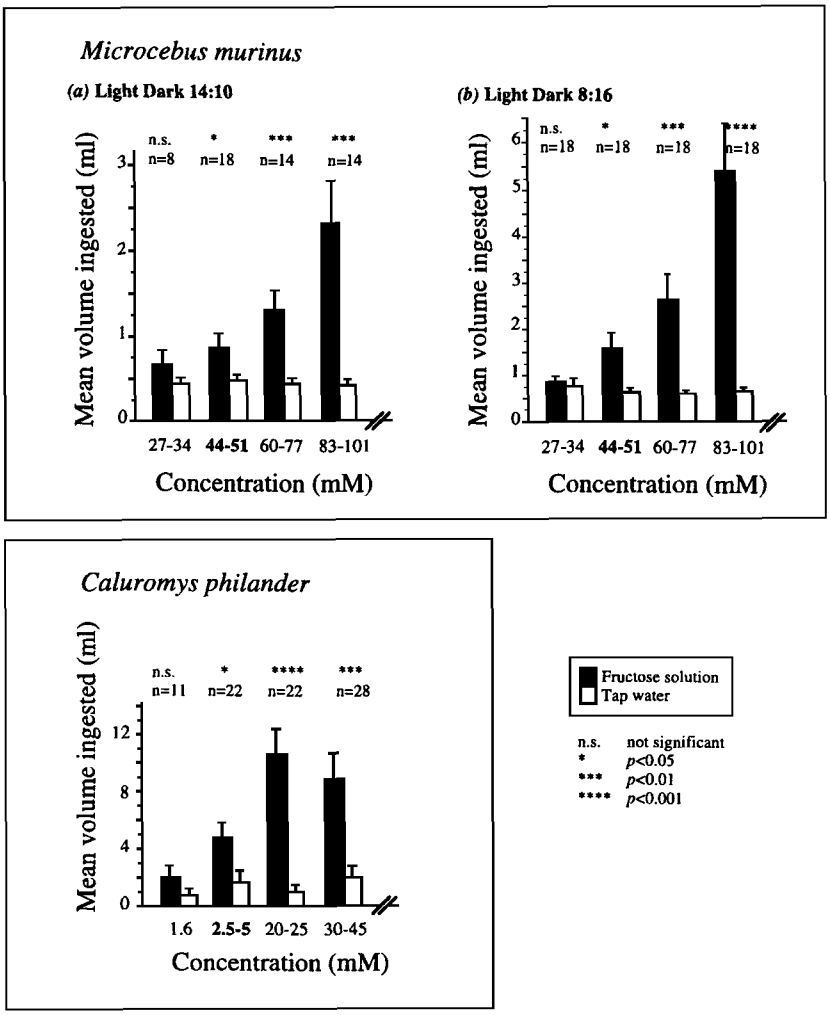

FIG. 1. Near-threshold ingestive responses toward fructose solutions and tap water of *Microcebus murine* males (upper panel) and *Caluromys philander* males and females (lower panel) in a two-bottle test. In *M. murinus*, tests were performed during the period of long daylength (a) and short daylength (b). Results have been grouped as increasing concentration intervals, the threshold being indicated in bold characters. The probability (*P*) that the mean difference of consumption between the sweet solution and tap water is significant is shown for each interval. The number (*N*) of individual responses may be lower than the total number (or multiple) of subjects (see Methods and Materials), but individual animals enter equally into the calculation of *P* for each block of concentrations.

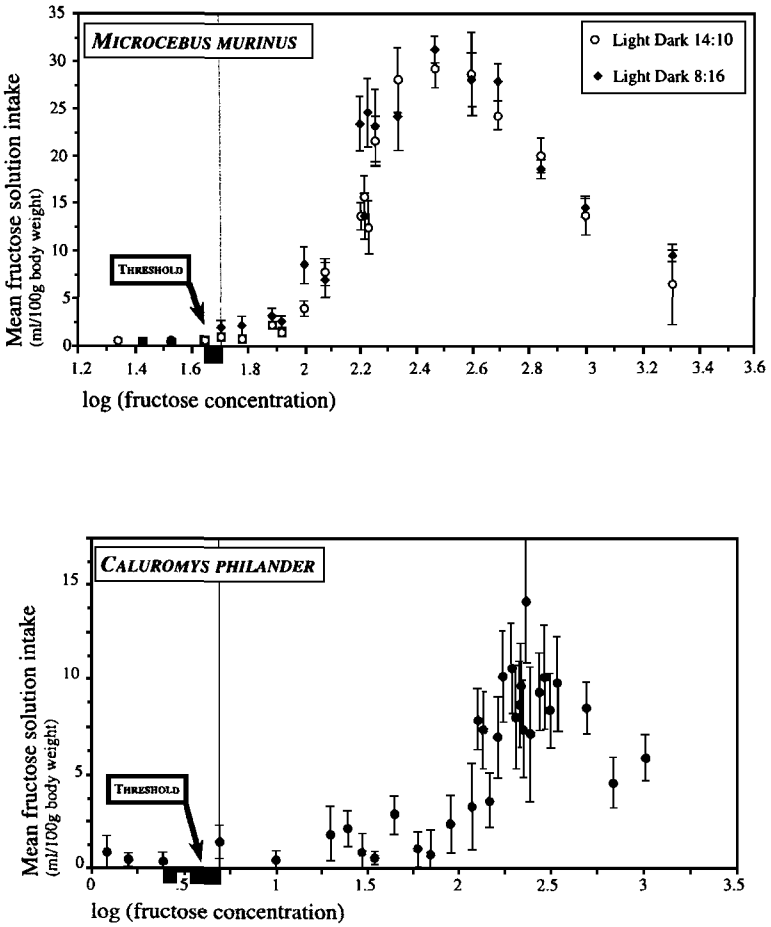

FIG 2. Profiles of suprathreshold responses to fructose solutions in *Microcebus murinus* (upper panel) and *Caluromys philander* (lower panel) recorded in a two-bottle test. In *M. murinus*, the figure illustrates responses obtained during long daylengths and short daylengths, respectively. Water intake is not indicated for better clarity. Mean liquid intake is expressed per unit body weight to control for individual differences.

cumulated concentrations until reaching its highest value (corresponding to solutions at 220–300 mM in *Microcebus* according to the individual). Cumulating data at higher concentrations yielded poorer values of the coefficient of determination because of departure from linearity in the rising limb of the profile. Slope B was obtained in a similar way, with data from 300 to 500 mM, according to the value of individual r^2, up to a concentration of 2 M.

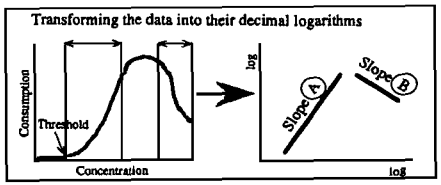

TABLE 1. RELATIONSHIP AMONG PHOTOPERIOD CHANGES, INDIVIDUAL BODY WEIGHT AND SUPRATHRESHOLD RESPONSES TO FRUCTOSE SOLUTIONS IN *Microcebus murinus*[a]

	Body weight (g)[b] (variation during test)		Slope A		Slope B	
Animal	LD	SD	LD	SD	LD	SD
4αd314	97 (−18%)	110 (+15%)	2.83	1.61; *	−0.94	−0.62; ns
4αd315	91 (+3%)	86 (+10%)	2.46	2.16; ns	−0.88	−0.77; ns
3αc17	123 (+6%)	128 (+3%)	2.20	2.42; ns	−1.16	−0.76; ns
2δg3	80 (−11%)	90 (+7%)	2.97	2.96; ns	−0.80	−0.67; ns
871bae	74 (+4%)	128 (+26%)	1.91	2.42; ns	ND	−0.77
883bcb	66 (−3%)	100 (+28%)	3.22	3.14; ns	ND	−0.81
901be	75 (+1%)	86 (+43%)	2.14	1.67; ns	ND	−0.71
811ddv	54 (−4%)	82 (+52%)	2.52	2.40; ns	ND	−0.89
920d	76 (+6%)	116 (+26%)	3.95	3.03; ns	ND	−0.87
Mean ± SD	82 ± 20 (−2% ± 8%)	103 ± 18 (+23% ± 16%)	2.69 ± 0.63	2.42 ± 0.57	−0.95 ± 0.15	−0.76 ± 0.09

[a]The method applied for logarithmic transformation of each bell-shaped curve (represented as the mean in Figure 2) into regression lines with positive and negative exponents (referred to as slope A and slope B, respectively) is described in the text. Whereas body weights are generally lower in the period of long daylength (LD) than in the period of short daylength (SD), neither slopes A (with one exception) nor slopes B vary significantly between the two seasons. The F test probability of a significant seasonal difference is indicated as *: $P \leq 0.05$; ns: not significant.

[b]Body weight at the end of the experiment. ND: not provided due to the lack of sufficient data at high concentrations.

Individual profiles and body weights are detailed in Table 1. In eight of nine animals, slopes A average 2.56 ± 0.29 and do not vary significantly between each season (with r ranging between 0.85 and 0.99). Only four animals could be used to compare slopes B at both period, and there was no significant variation ($0.87 < r < 0.999$). After grouping all individual results, slopes B average was −0.821 ± 0.14. The lack of seasonal variation of both the threshold and suprathreshold slopes occurs independently of the fact that the animals gained weight during the fattening period of short daylength (+23% on average at the end of the tests; $t = 4.202$, $P < 0.01$). As shown in Table 1, interindividual differences exist in the steepness of the slopes in each period. These differences appear to be larger within slopes A (up to a factor of 2) than within slopes B. There is no correlation between steepness of slope A and either body weight or body weight variation during the experiment at 14 hr daylength ($P > 0.6$, df 8 in both cases), or at 8 hr daylength ($P > 0.5$, df 8).

In the woolly opossum (Figure 2), individual data were averaged, although a large number of tests was performed to compensate for some variability in the responses, and the regression lines were calculated as for mouse lemurs on data above the threshold. There was no statistical difference between mean slope A of males and that of females (0.976 and 0.985, respectively; $F = 0.00088$; $df = 46$). By contrast, a highly significant difference occurs between *Caluromys* and *Microcebus*, with woolly opossum males increasing their fluid intake more progressively than lemur males ($F = 5.461$; $P < 0.05$; $df = 35$). Clustering the data of *Caluromys* yields a slope of 0.759 ± 0.215 ($r = 0.83$). The statistical value of slope B cannot be ascertained given the limited data at high concentrations, but an indicative calculation gives −0.6 as a figure.

Ingestive Responses to Mixtures of Fructose and Tannic Acid Solutions. In *Microcebus*, the depressing effect of tannic acid on binary solution intake is significant, compared with pure fructose solutions (Figure 3) when the concentration reaches 0.42 mM for the low sugar mixture and 0.31 mM for the high sugar solution. The fructose–tannin mixtures were nevertheless all significantly preferred over water. In *Caluromys*, the mixtures are less attractive than pure sugar solutions above tannin concentration thresholds of 0.25–0.63 mM (Figure 3). The binary solutions remained preferred over water until they contained 0.25, 1.27, and 7.62 mM of tannic acid as the fructose concentration increased.

In both *Caluromys* and *Microcebus*, the mixtures are not fully deterrent once the inhibitory threshold is reached. As expected, a given perceived tannin concentration has more depressive effects on low sugar solutions than on high concentrations. In *Caluromys*, a 50% depressing effect is obtained for a tannin concentration at 0.25 mM for the low sweetness solution versus 0.63 mM for the moderately sweet solution and by adding more than 1.27 mM to the concentrated sugar solution. As represented for the high sugar solution (Figure 3, insert), the inhibition is progressive, following an asymptotic curve up to 97%. The curve

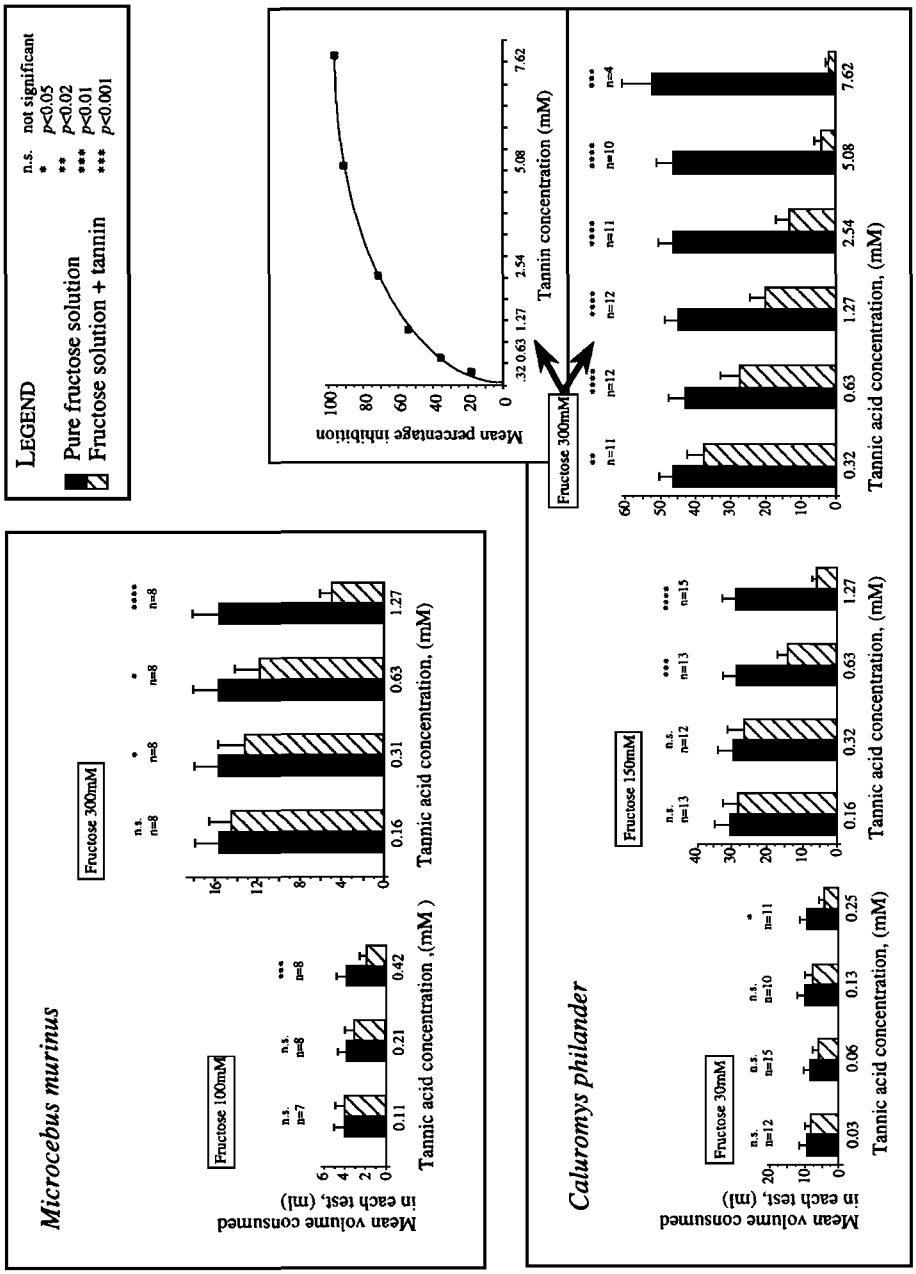
LEGEND
Pure fructose solution
Fructose solution + tannin
n.s. not significant
* p<0.05
** p<0.02
*** p<0.01
*** p<0.001
Microcebus murinus
Fructose 300mM
Fructose 100mM
Mean volume consumed in each test, (ml)
Tannic acid concentration ,(mM)
Mean percentage inhibition
Tannin concentration (mM)
Caluromys philander
Fructose 150mM
Fructose 30mM
Tannic acid concentration, (mM)

FIG. 3. Inhibition thresholds of tannic acid in mixture with fructose solutions and depressing effect on liquid consumption in *Microcebus murinus* and *Caluromys philander*. The inhibition threshold is defined as the lowest tannin concentration for which the mean difference of consumption between the sweet solution and the mixture is significant ($P \leq 0.05$ in a paired-sample t test). A plot of the mean percentage inhibition as tannin concentration increases is illustrated for *C. philander*, with 300 mM fructose solution as the reference. The concentration of tannic acid is expressed in millimoles per liter of sweet solution.

was found to be best fitted by a model of Monod [nonlinear regression with $r^2 = 0.99$; $y = (a \times x)/(b + x)$], with the corresponding equation $y = X/(1.18 + X)$ with $a = 1$ and $X = x - 0.25$.

DISCUSSION

A previous study of *Microcebus* males showed that the taste preference threshold for sucrose, recorded from the two-bottle test, varied seasonally in the presence of a standard array of foods (Simmen and Hladik, 1988). However, the lesser mouse lemur remained able to discriminate low concentrations, as revealed by the conditioned taste aversion paradigm (Hellekant et al., 1993b). Here, when prevented from feeding on supplementary sources during taste tests, males displayed no seasonal variation of either the taste threshold or suprathreshold responses to fructose. This indicates that, in the context of limited food supply, gustatory potentialities are fully expressed (i.e., near-threshold concentrations are perceived as palatable). The similarity of thresholds for sugars determined with electrophysiological and behavioral experiments in different simian species (Ogawa, 1972; Glaser and Hellekant, 1977) and the lack of variation observed here in *Microcebus* despite marked seasonal endocrinological and behavioral fluctuations make the two-bottle test a reliable method to estimate discrimination thresholds under the above described testing procedure.

The lesser mouse lemur and the bare-tailed woolly opossum exhibit many similar ecological characteristics, among which is a frugivorous/insectivorous tendency. The data indicate that their perception of sugars, although differing in some respect, is globally consistent with diets based on fruits and with the need for detecting variations in biochemical content of foods. The ability to taste fructose can be appreciated by referring to data obtained on nonhuman primates with a two-bottle test. With a threshold at 44–51 mM, *Microcebus murinus* is relatively insensitive, as could be predicted by the negative correlation between taste thresholds for sugars (sucrose and fructose) and body weight found in primates. Thresholds for fructose vary from about 14 to 66 mM across primate species (Simmen and Hladik, 1998). In contrast, *Caluromys* appears a much better taster (2.5–5 mM). The profiles of suprathreshold responses to sugars have been reported to approximate bell-shaped curves in rats (Richter and Campbell, 1939; Pfaffmann, 1960), squirrel monkey (Michels et al., 1988), and man [in terms of affective perception (Moskowitz et al., 1974)]. In this study, the profiles follow the same pattern, but present a dissymmetry around the point of inflection. The two species differed in that the rising limb of the profile was much more progressive in *Caluromys* than in *Microcebus*.

A species' ability to taste low sugar concentrations and the palatability of above-threshold solutions likely have some consequences on the categorization

of natural food resources and, in turn, on feeding selectivity. Whereas in the opossum a wide range of foods, including those with low amounts of soluble sugars, are potentially palatable, stimuli presenting only slightly different sweetness intensities appear equally attractive. Conversely, in the lesser mouse lemur, subtle differences of concentration—as occurs, for instance, in a bunch of fruits at different stages of ripeness—may be associated with distinct sensory rewards. Field observations provide evidence that fruits selected by *Caluromys* have sugar contents of up to 86% of dry weight, i.e., roughly 305 g/liter in fruits containing 74% water (Atramentowicz, 1988). This is above the highest concentrations provided in this study and far above the threshold (about 1 g/liter). Accordingly, sensory reward probably is a main immediate component of fruit-eaters' motivation for selecting a particular array of ripe fruits within a biochemically changing environment.

It is interesting to note that the downward portion of the ingestion profiles resulted in constant energy input. Within this range of concentrations, energy intake per unit body weight and time (more than 20 cal/g/hr in both species) is beyond basal metabolic needs [4–7 cal/g/hr in lesser mouse lemur males (Perret, 1980); 3–3.5 cal/g/hr in woolly opossums (Atramentowicz, unpublished data)] and expected field metabolic rates. It is not clear whether these sugar solutions conditioned an aversion (e.g., anorexia due to hypertonicity of the solutions) or were simply less pleasant to the animals. In humans, very sweet solutions are considered less pleasant (Moskowitz et al., 1974). Furthermore, the consumption of preferred foods with high sucrose concentrations is lower than that of the same foods with lower sugar content (Lucas and Bellisle, 1987). At high concentrations, the measure of food intake does not adequately reflect pleasantness since the various inhibition processes involving oral satiation and monitoring of caloric density may take place rapidly after ingestion (Vanderweele, 1974; Le Magnen, 1987; Rolls et al., 1989).

In many plant species that have evolved generalized chemical defenses, tannin concentrations tend to decrease as fruits ripen and the amount of soluble sugars increases (Goldstein and Swain, 1963; Wrangham and Waterman, 1983). Our testing procedure, designed to mimic such variations, showed that both species were similarly affected by the addition of tannic acid to sugar solutions of various concentrations. Hellekant et al. (1993a) report that the chorda tympani proper nerve of *Microcebus murinus* responds to a pure gallotannin concentration of 0.2 mM but not 0.075 mM. Using the same batch of the gallotannin, we found that slightly higher concentrations depressed the ingestion of low and high fructose solutions in *Microcebus*. Given that the perceived intensity of tannic acid is decreased by sweetness (Lyman and Green, 1990), the results of the two studies can be considered largely concordant. In *Caluromys*, the threshold values lay within the same range (0.25–0.63 mM).

There is evidence that tannin-rich foods are consumed by a variety of mam-

mal species (Furstenburg and van Hoven, 1994; Marks et al., 1988). Immature fruits eaten by chimpanzees contain 5% condensed tannin on average (roughly 12 g/liter assuming 80% moisture in fruits) (Wrangham and Waterman, 1983), a concentration that almost totally inhibits the ingestion of sweet solutions in *Caluromys* and *Microcebus*. The inhibition curve of tannic acid indicated some degree of tolerance, consistent with field observations that fruits consumed by the lesser mouse lemur contain significant amounts of condensed tannins [2% on average according to Ganzhorn (1988)]. Tolerance levels were dependent upon the concentration of fructose, which corroborates the idea of a trade-off between acceptable levels of tannins and nutrient content, probably mediated by oropharyngeal sensations.

It is remarkable that similar effects have been recorded in other small species feeding on fruits and gums. In Goeldi's monkey and marmoset, which exhibit taste thresholds for fructose around 30 mM, consumption is decreased by more than 50% when 0.25 mM tannic acid is added to near-threshold sugar concentrations or when 5 times more concentrated tannin levels are added to high sugar concentrations (Simmen, 1994). In light of the experimental results, combined with available data on food chemistry, it would seem that small frugivorous mammals show some convergence in their responses toward tannin and tolerate low concentrations. Since larger species of similar dietary grade tend to include foods with higher tannin contents in their diet, the possibility of an allometric scaling of taste sensitivity toward tannins must be tested, keeping in mind that the astringent taste eventually covers several taste qualities or subqualities.

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A MAJOR FUNGITOXIN FROM *Pachira aquatica* AND ITS ACCUMULATION IN OUTER BARK

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Abstract—A major fungitoxin in the root of *Pachira aquatica* (Bombacaceae) was isolated and identified as isohemigossypolone. Quantitative analysis of isohemigossypolone in different parts of *P. aquatica* revealed that the outer bark of the swollen trunk accumulated a large amount of the fungitoxin. The concentration of the substance was more than 1 mg/g fresh weight in the outer bark tissues. Pure isohemigossypolone showed clear antifungal activity against *Pythium ultimum* at a minimum dose of 10 μg/disk, and it may function as a defensive chemical to protect storage tissues of *P. aquatica*.

Key Words—*Pachira aquatica*, Bombacaceae, isohemigossypolone, cadinane sesquiterpene, naphthoquinone, chemical defense.

INTRODUCTION

The genus *Pachira* of Bombacaceae has two species, *P. aquatica* and *P. macrocarpa*, both distributed throughout Central America. *P. aquatica* also grows into a medium-tall tree, and the bottom of the trunk is swollen to store carbohydrates and water (Figure 1). Because of the unique shape and its drought-tolerant nature, *P. aquatica* is popular in Japan as an indoor ornamental tree.

In the course of chemical study of plant defensive mechanisms, we have investigated the antimicrobial substances of plants. When we screened *P. aquat-*

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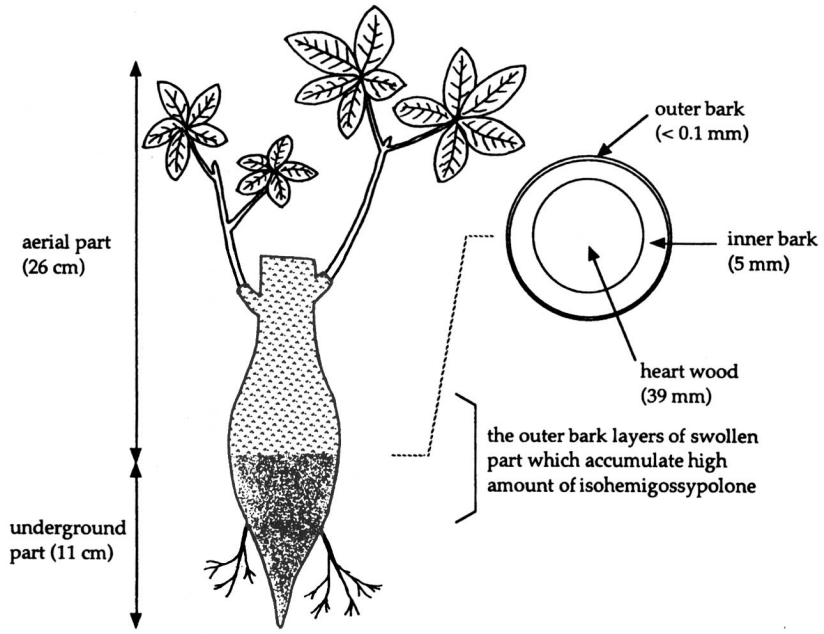


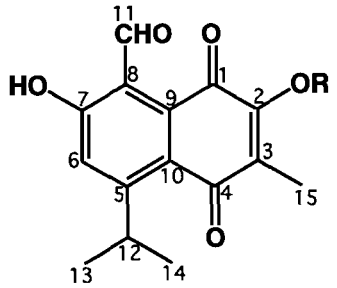
FIG. 1. A diagram of *Pachira aquatica* illustrating the swollen underground part that accumulates isohemigossypolone.

ica, significant antifungal activity was detected only in extract of the swollen underground part. The major fungitoxin was identified as isohemigossypolone (**1**) (Figure 2) by spectroscopic analyses, including NMR. Isohemigossypolone has been found in some other Bombacaceae (Seshadri et al., 1971, 1973; Sankaram, 1981). In this report we describe structural and quantitative analyses of isohemigossypolone, demonstrate its accumulation in the swollen underground part of the plant and its toxicity, and discuss the significance of it in chemical defense of *P. aquatica*.

METHODS AND MATERIALS

Instrumentation. ^{1}H- and ^{13}C NMR spectra were measured with a Jeol EX 270 at 270 and 68 MHz or with a Bruker AM500 at 500 and 125 MHz (HMBC, HMQC), with TMS as the internal standard. EI-MS spectra were measured with a Jeol DX-500.

Plants. Pachira aquatica used in this study was purchased from a plant shop in Sapporo. Three plants of approximately the same size, 20–30 cm height



1 R=H isohemigossypolone
2 R=Me 2-*O*-methylisohemigossypolone

FIG. 2. The structures of isohemigossypolone and 2-*O*-methylisohemigossypolone.

and 12–15 cm in girth of trunks, were chosen as test speciments for comparing antifungal activity. These plants were conditioned for a few days in the laboratory prior to experiments.

Antifungal Assay. The underground part (25 g) and leaves (5 g) of *P. aquatica* were extracted with excess ethyl acetate (EtOAc), and the residues were reextracted with methanol (MeOH). Each extract was concentrated and divided into two parts. One was diluted to contain 50 mg solute/ml, and the other to contain solute equivalent to 1 g fresh weight (1 g fw) of the tissues per milliliter. Each solution (25 μl) was chromatographed on TLC plates (0.25 mm, Kieselgel 60 F_{254} Merck). The solutions charged on TLC plates contained 1.25 mg of the extracts and those from 25 mg of the fresh tissues, respectively. The TLC plates were developed in hexane–EtOAc (2 : 1). Ten-fold diluted solutions (25 μl each) were also chromatographed. As a preliminary antifungal test, a spore suspension of *Cladosporium herbarum* AHU 9262 was sprayed over the developed TLC plates, and incubated at 25°C under high humidity conditions for three days (Homans and Fuchs, 1970). Quantitative antifungal assay of pure isohemigossypolone (**1**) also was done in the same manner. Acetone solutions of recrystallized **1** (1000, 500, 250, 100, 50, 25, and 10 μg/ml) were prepared and charged on TLC plates (25 μl).

A paper disk assay for antifungal activity was done as follows: Recrystallized **1** was dissolved in EtOAc at several concentrations (4000, 400, 40, and 4 μg/ml), and 25 μl was placed on paper disks (8 mm diameter). Thus, the amount of the test compound on a disk was 100, 10, 1, and 0.1 μg, respectively. The disks were dried in vacuo to remove the solvent and were put on 2% potato-dextrose (Difco) or nutrient-broth (Nissui) agar (10 ml) plates; then 0.5% agar (10 ml) containing cells of *Saccharomyces cerevisiae* or *Escherichia coli* or chopped hyphae of *C. herbarum*, *Pythium ultimum*, *Rhizoctonia solani*,

or *Aspergillus flavus* was layered over each plate. Plates were incubated at 25°C under high humidity conditions for one to two days. The growth inhibition value was expressed by the diameter (millimeters) of the inhibitory zone.

Isolation of Isohemigossypolone (1) and 2-O-Methylisohemigossypolone (2). The whole underground part of *P. aquatica* (82.1 g fw) was extracted with EtOAc (600 ml) for three days, and the extract (210.7 mg) was applied to a silica gel (SiO_2) column containing 15 g of Wako-gel C-200 in chloroform ($CHCl_3$), and fractions were eluted with $CHCl_3$–MeOH mixtures. Fractions collected (10 ml each) were as follows: $CHCl_3$ only (fractions 1–3), $CHCI_3$–MeOH 50 : 1 (fractions 4–8), 50 : 2 (fractions 9–11), and 50 : 4 (fractions 12–14). Compound **1** was eluted in fractions 5–9 while **2** was in fractions 3 and 4. The fractions containing each compound were combined and concentrated to yield an orange solid (37.2 mg) and a brown oil (30.2 mg), respectively. Compound **1** was recrystallized from a mixture of hexane–$CHCl_3$ to give pale yellow prisms (26.2 mg).

Identification and Structural Analyses of Isolated Compounds. Isohemigossypolone (**1**) crystallized as pale yellow prisms. Mp 168–169.5°C. R_f in hexane–EtOAc (2 : 1), 0.71, EI-MS (*m/z*, %): 274 (M^+, 100), 259 ($M-Me^+$, 61), 257 ($M-OH^+$, 30), 246 ($M-CO^+$, 15), 231 [$M-CH_2(CH_3)_2^+$, 12]. ^1H NMR δ (500 MHz, $CDCl_3$, HH-COSY): 12.45 (s, 7-OH), 10.83 (s, H-11), 7.33 (s, H-6), 7.08 (s, 2-OH), 4.32 (sept., J = 6.8 Hz, H-12), 2.08 (3H, s, H_3-15) and 1.28 (6H, d, J = 6.8Hz, H_3-13 and H_3-14). Proton signals at δH 12.45 and 7.08 disappeared when D_2O was added. ^{13}C NMR δ (125 MHz, $CDCl_3$, HMQC): 197.7 (11-CH), 186.2 (4-C), 183.4 (1-C), 164.6 (7-C), 161.5 (5-C), 151.2 (2-C), 132.5 (9-C), 124.7 (10-C), 122.9 (6-CH), 121.2 (3-C), 116.4 (8-C), 29.9 (12-CH), 23.5 (13- and 14-CH_3), and 9.0 (15-CH_3). HMBC (125 MHz, $CDCl_3$), protons → (carbons): 12.45 → (164.6, 122.9, and 116.4), 10.83 → (116.4), 7.33 → (124.7, 116.4, and 29.9), 7.08 → (151.2 and 121.2), 4.32 → (161.5, 122.9, and 23.5), 2.08 → (186.2, 151.2, and 121.2), and 1.27 → (161.5 and 29.9). NOESY (500 MHz, $CDCl_3$), proton → protons: 1.27 → 7.33 and 4.32.

2-*O*-Methylisohemigossypolone (**2**) was a yellow syrup. Its R_f in hexane–EtOAc (2 : 1) was 0.87. EI-MS (*m/z*, %): 288 (M^+, 100), 273 ($M-Me^+$, 37), 271 ($M-OH^+$, 19), 256 (11), 245 (66), 95 (17) and 81 (17). ^1H NMR δ (500 MHz, $CDCl_3$): 12.34 (s, 7-OH), 10.47 (s, H-11), 7.26 (s, H-6), 4.29 (sept., J = 6.9 Hz, H-12), 4.05 (3H, s, 2-OMe), 2.06 (3H, s, H_3-15) and 1.20 (6H, d, J = 6.9 Hz, H_3-13 and H_3-14).

Quantification of Isohemigossypolone. Recrystallized isohemigossypolone was dissolved in EtOAc to prepare solutions containing known amounts of 1000, 500, 250, 100, 50, 25, and 10 μg/ml. Methyl palmitate dissolved in hexane (10 mg/100 ml) was used as the internal standard. To each 100 μl of **1** an equal volume of the standard solution was added, and the resulting mixture was concentrated and redissolved in 20 μl of EtOAc to apply for gas chromatographic (GC) analysis.

The GC conditions were as follows. The column was a high-resolution capillary column with OV-1 Bonded, 25 m × 0.3 mm (GL Science Inc.). The oven temperature was 150°C for 1 min and was programmed from 150°C to 260°C at a rate of 5°C/min. Compounds were detected by a flame ionization detector (FID). Retention times (R_t, minutes) for the internal standard and **1** were 7.21 and 10.21, respectively. A curve of peak ratios of **1**/internal standard plotted as a function of quantity of **1** was linear between 1 and 100 μg of **1**.

Each part (aerial parts: outer bark, 0.37 g; inner bark, 14.61 g; heartwood, 13.57 g; underground parts: outer bark, 0.33 g; inner bark, 3.08 g; heartwood, 3.84 g) was chopped well and extracted with MeOH for three days. Each extract was partitioned between EtOAc and water, and the EtOAc layers were dried over Na_2SO_4, concentrated, and applied to a Sep-Pak column (Waters, 3 ml Silica in $CHCl_3$), and eluted with a mixture of $CHCl_3$–MeOH (9 : 1, v/v). After compound **1** was eluted thoroughly, 100 μl of the standard solution was added and the solution was concentrated to 20 μl of EtOAc for GC analysis.

RESULTS AND DISCUSSION

TLC bioautography with *C. herbarum* as a test fungus resulted in a single antifungal spot at a dose rate corresponding to 0.125 mg of the extract or extracts from 100 mg fresh tissue of underground parts. Thus, we focused on this antifungal substance, which had an R_f of 0.76 in hexane–EtOAc (2 : 1). Silica gel column chromatography of the EtOAc extract and successive recrystallization of the antifungal principle yielded 26.2 mg of fine prisms. Several spectroscopic analyses indicated a highly unsaturated sesquiterpene nature. EI-MS gave M^+ at m/z 274, but the fragmentation pattern was not very helpful. ^1H NMR spectra revealed the presence of an aldehyde group due to δH 10.83 and an allylic methyl group (δH 2.08); a pair of isopropyl protons [δH 1.28 (6H) and 4.32 (1H)] was also visible. D_2O addition resulted in the disappearance of two singlet peaks at δH 12.45 and 7.08, indicative of signals of a phenolic groups, and the former indicated an intramolecular hydrogen-bonding. Based on a literature search, structures for hemigossypolone (Seshadri et al., 1971; Bell et al., 1975; Gray et al., 1976) and isohemigossypolone seemed appropriate. HMBC analyses, however, showed the compound to be isohemigossypolone (**1**), proposed by Sankaram et al. (1981). Some correlations (between 7-OH and 6-CH, and 3-C and 2-OH) and NOE between 7-H and isopropyl methyl signals were sufficient only for structure **1**, together with consideration of the isoprene rule. A minor antifungal substance found in the column fractions that eluted a little earlier than those containing **1** was identified as 2-*O*-methylisohemigossypolone (**2**).

In TLC bioautography, pure compound **1** showed a minimum inhibitory dose (MID) at approximately 1.6 μg/cm^2. In the paper disk assay, **1** showed

growth inhibitory activity against *P. ultimum* at 10 μg/disk. Compound **1** was active also against *C. herbarum* and *S. cereviciae* at 1 μg/disk, but showed no activity against *E. coli*, *A. flavus*, and *R. solani* at 10 μg/disk. Based on these results, carbohydrate-requiring microorganisms are more likely to show high susceptibility.

According to the quantitative analyses, a large amount of **1** is accumulated in the outer bark, especially in bark tissue located underground and surrounding the storage tissues. In contrast, the inner bark, including the green layer, and also the soft heartwood, which is filled with a large amount of water and polysaccharides and occupies most parts of the underground tissues, contained no significant amount of compound **1** (Table 1). The concentration of **1** in the outer bark was about 2 mg/g and was about 2×10^2 times higher than the effective minimum concentration of the compound on the TLC bioautography. Since comopund **1** possesses the quinone partial structure that causes the Michael addition reaction to show nonspecific cytotoxicity (Hellerman and Perkins, 1934; Muguerrian, 1955; Owens and Novotny, 1958), living plant cells would be damaged by this sesquiterpene. The segregated deposition of this physiologically active compound, however, generally seems to be an effective way to avoid damage on the living plant tissue itself. Consequently, high accumulation of **1** at the outer barks appears to be a way in which *P. aquatica* deters self-intoxication from endogenous **1** and still has a chemical defense. A similar topical deposition of naphthoquinone-type cadinane sesquiterpenes has been observed in cotton, which concentrates the chemicals in exudates from its glandular trichomes where they function as antifeedants against caterpillars on cotton (Lukefahr et al., 1966; Bell et al., 1978).

TABLE 1. QUANTIFICATION OF ISOHEMIGOSSYPOLONE (**1**) IN SELECTED TISSUES OF *Pachira aquatica*

Tissue or part of trunk	Amount of **1** (μg/g fresh weight)[a]		
	A	B	C
Underground parts			
Outer bark	1709	2143	1832
Inner bark	ND	ND	1.1
Heartwood	ND	ND	1.1
Aerial parts			
Outer bark	111	544	546
Inner bark	ND	ND	<0.1
Heartwood	ND	ND	ca. 0.1

[a]The reliable level of quantitative detection by GC analysis is more than 0.1 μg/g fresh weight. <0.1: the content is less than 0.1 μg/g fresh weight, but detectable. ND: not determined.

The regions where *P. aquatica* are distributed are highly diversified in ecosystems, and the soil possesses a high potential for pests. In such circumstances, toxic chemicals accumulated in outer bark may function as an effective chemical protector. A similar accumulation of stylbenoids, also known as defensive compounds, has been found in the outer bark of the pine tree *Picea glehnii* (Sano and Sakakibara, 1977).

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RELATIVE CONFIGURATION OF A δ-LACTONE ISOLATED FROM MANDIBULAR GLAND SECRETION OF *Calomyrmex* sp. MALES

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Abstract—The relative configuration of a δ-lactone isolated from the mandibular gland extracts of *Calomyrmex* sp. males has been determined to be (3*SR*,5*RS*,6*SR*)-3,5,6-trimethyltetrahydropyran-2H-one after the synthesis of the four possible racemates and comparison of their mass spectra and gas chromatographic properties with those reported in the literature for the natural product.

Key Words—*Calomyrmex* sp. males, mandibular gland secretion, 3,5,6-trimethyltetrahydropyran-2-ones, synthesis.

INTRODUCTION

In studies with Australian desert ants (*Calomyrmex* sp.), Brough reported that a complex mixture of substances was secreted by the mandibular glands of disturbed workers, and the mixture was shown to have an important role in alarm communication and defense (Brough, 1976, 1977, 1978). Later, the secretion from mature workers was found to have antimicrobial activity when tested against selected soil microorganisms (Brough, 1983).

The mandibular gland of *Calomyrmex* sp. workers is extremely well developed, and the color of its secretion varies with age from white in the youngest workers to deep orange in the mature ones. However, a colorless secretion occurs in *Calomyrmex* sp. males, suggesting different roles for the mandibular gland secretion in workers and males (Brough, 1977). Considering that the

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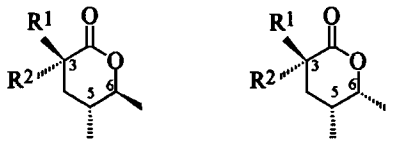


1a R^1=H, R^2=Me **1c** R^1=H, R^2=Me
1b R^1=Me, R^2=H **1d** R^1=Me, R^2=H

FIG. 1. Structures of the four possible racemates of 3,5,6-trimethyltetrahydropyran-2H-one.

mandibular gland secretions of other hymenopteran males function as sex attractants, a similar role in *Calomyrmex* sp. males is possible (Lloyd et al., 1975; Brand et al., 1973).

Brown and Moore (1979) studied the chemical constituents of the mandibular gland secretion of workers, gynes, and males by gas chromatography. While the mixture secreted by workers was shown to be composed of 6-methylhept-5-en-2-one and its corresponding alcohol, terpenoids, and pyrazines, the analysis of the pentane extract of male heads indicated two major components in approximately equal amounts: nerol and a δ-lactone that was presumed to function as a sex pheromone. It was 3,5,6-trimethyltetrahydropyran-2H-one (Figure 1) based upon mass spectrum analysis and preparation of a mixture of its four possible racemates (**1a–d**) from the condensation of 1-chloro-2-methylbutan-3-one and sodium diethyl methylmalonate. However its relative configuration was not established.

METHODS AND MATERIALS

^{1}H NMR spectra were recorded in $CDCl_3$ solution at 300 MHz and ^{13}C NMR spectra in $CDCl_3$ solution at 75.2 MHz (unless otherwise noted) with a Varian Gemini 2000 or a Bruker AC-300P instrument. Chemical shifts are expressed in parts per million relative to tetramethylsilane followed by multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; qt, quintet, m, multiplet), coupling constant, and number of protons. Infrared spectra were recorded on a Perkin-Elmer 399B or 1600 series spectrophotometer. Mass spectra were obtained via electron impact (30 eV) on a Varian MAT 311A spectrometer. Elemental analyses were performed at Instituto de Quimica, Unicamp. High-resolution mass spectra were kindly provided by Professor Armin de Meijere, Georg-August Universität, Göttingen, Germany.

GC analyses were carried out in capillary columns (30 m × 0.53 mm) with 1% phenylmethylsilicone (HP-1) or cross-linked polyethylene glycol (Carbowax

20 M) as stationary phases. GC-MS analyses were performed on a Hewlett-Packard 5890 series II gas chromatograph coupled to a MSD 5970 mass detector equipped with a capillary column (Carbowax 20 M, 25 m × 0.20 mm × 0.33 μm).

Column chromatography was performed with silica gel (70–230 mesh), except when stated otherwise, and reactions were monitored by TLC (plates from Macherey-Nagel, Germany).

Chemicals. Ether and tetrahydrofuran were treated with sodium–benzophenone and distilled immediately prior to use. Dichloromethane, triethylamine, diisopropylamine, and benzene were treated with calcium hydride and distilled immediately prior to use. Acetaldehyde, oxalyl chloride, acetic, and propionic anhydride were distilled prior to use. The remaining reagents employed were purchased from commercial suppliers and used without further purification. The reactions involving anhydrous solvents were carried out under argon atmosphere.

*(2SR,3SR)-2′,6′-Di-*tert*-butyl-4′-methylphenyl-3-hydroxy-2-methyl-butanoate* (**3**). To a LDA solution prepared from diisopropylamine (1.1 ml, 7.9 mmol) and 1.6 M *n*-BuLi in hexane (4.5 ml, 7.2 mmol) in THF (3.0 ml), we added dropwise at −78°C a solution of ester **2** (2.0 g, 7.2 mmol) in THF (2.0 ml). After 1 hr at −78°C, a solution of acetaldehyde (1.03 g, 23.5 mmol) in THF (2.0 ml) was added and the reaction stirred for 30 min and then quenched by the addition of satd. NH_4Cl (7.2 ml). The reaction mixture was diluted with ether (10 ml), and the organic phase was washed with 1% aq. HCl (3 × 5.0 ml), satd. $NaHCO_3$ (3 × 5.0 ml), and satd. NaCl (3 × 5.0 ml). The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude mixture was purified by recrystallization from hexane to afford **3** (1.38 g, 4.32 mmol), as a colorless solid in 60% yield.

Mp: 109.9–110.7°C (found: C, 75.51%; H, 10.39%; $C_{20}H_{32}O_3$ requires: C, 74.96%, H, 10.06%). IR (KBr): 3360 and 1785 cm^{-1}. ^1H NMR: δ 1.30 (d, J = 6.3, 3H, CH_3-2), 1.32 [s, 9H, $-C(CH_3)_3$], 1.33 [s, 9H, $-C(CH_3)_3$], 1.47 (d, J = 7.5, 3H, CH_3-1), 2.32 (s, 3H, CH_3-4′), 2.72 (q, J = 7.5, 1H, CH-3), 3.70 (d, J = 3.30, 1H, OH), 4.02–4.08 (m, 1H, CH-2), 7.13 (s, 2H, CH-3′ e 4′). ^{13}C NMR: δ 13.1, 20.4, 21.5, 31.4, 31.5, 35.2, 35.3, 47.7, 68.9, 127.0, 127.2, 134.8, 141.8, 142.0, 145.8, 176.3. MS (*m/z*): 220 (100%), 205 (100%), 103 (23%), 73 (25%), 57 (63%), 45 (97%).

*(2RS,3SR)-1-*O-p-*Toluenesulfonyl-2-methyl-1,3-butanediol* (**4**). To a suspension of $LiAlH_4$ (0.20 g, 5.3 mmol) in THF (15 ml) at 0°C was added a solution of **3** (0.86 g, 2.7 mmol) in THF (8 ml). The reaction was stirred overnight at room temperature and then treated with water (0.20 ml), 10% aq. NaOH (0.20 ml), and water (0.60 ml), successively. The organic layer was separated, the solids were washed with ether (2 × 10 ml), and the combined organic phase was evaporated under reduced pressure. The crude product was purified by column chromatography (1 : 1 hexane–ethyl acetate) to afford (*2RS,3SR*)-2-methyl-1,3-butanediol (0.20 g, 1.9 mmol) in 70% yield.

To a solution of the above mentioned diol (0.20 g, 1.9 mmol), Et_3N (0.29 ml, 2.1 mmol) and 4-*N*,*N*-dimethylaminopyridine (0.010 g) in CH_2Cl_2 (2.0 ml) at 0°C was added *p*-toluenesulfonyl chloride (0.38 g, 2.0 mmol). The reaction mixture was kept overnight at −15°C and then diluted with CH_2Cl_2 (10 ml) and washed with 1% HCl (2 × 3 ml), satd. $NaHCO_3$ (2 × 5 ml), and satd. NaCl (2 × 5 ml). The organic layer was dried over $MgSO_4$, evaporated under reduced pressure, and the crude product was purified by column chromatography (15% ethyl acetate in hexane) to afford **4** (0.44 g, 1.7 mmol), in 90% yield, as a colorless oil (found: C, 55.79%; H, 6.97%; $C_{12}H_{18}O_4S$ requires: C, 55.79%; H, 7.02%).

IR (film): 3422 (br), 1355 and 1175 cm^{-1}. ^1H NMR: δ 0.91 (d, J = 6.7, 3H, CH_3-2), 1.15 (d, J = 6.3, 3H, CH_3-4), 1.75–1.82 (m, 1H, CH-2), 2.02 (s, br, 1H, OH), 2.45 (s, 3H, CH_3-$ArSO_2$), 3.65–3.69 (m, 1H, CH-3), 4.05 (dd, J = 9.6 and 4.6, 1H, CH_2-1), 4.11 (dd, J = 9.8 and 5.5, 1H, CH_2-1), 7.35 (d, J = 8.1, 2H, Ar), 7.79 (d, J = 8.1, 2H, Ar). ^{13}C NMR: δ 13.2, 20.9, 21.6, 40.5, 68.6, 72.8, 128.1, 130.1, 133.3, 145.0. MS (*m*/*z*): 217 (8%), 173 (100%), 172 (50%), 155 (27%), 91 (88%).

*(2*RS,*3*SR*)-1-*O-p-*Toluenesulfonyl-3-*O*-propionyl-2-methyl-1,3-butanediol* (**5**). To a solution of **4** (0.18 g, 0.70 mmol) in CH_2Cl_2 (2.0 ml) was added Et_3N (0.15 ml, 1.1 mmol), 4-*N*,*N*-dimethylaminopyridine (0.010 g), and propionic anhydride (0.10 ml, 0.78 mmol). The mixture was stirred 30 min at room temperature and then diluted with CH_2Cl_2 (10 ml). The organic phase was washed with 1% HCl (2 × 5.0 ml), satd. $NaHCO_3$ (2 × 5.0 ml), and satd. NaCl (2 × 5 ml). The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by column chromatography (5% ethyl acetate in hexane) to afford **5** (0.19 g, 0.60 mmol), in 85% yield, as a colorless oil (found: C, 57.43%; H, 6.68%; $C_{15}H_{22}O_5S$ requires C, 57.30%; H, 7.05%).

IR (film): 1730, 1355 and 1170 cm^{-1}. ^1H NMR: δ 0.94 (d, J = 6.7, 3H, CH_3-2), 1.07 [t, J = 7.6, 3H, $-OC(O)CH_2C\mathbf{H_3}$], 1.15 (d, J = 6.4, 3H, CH_3-4), 1.95–2.10 (m, 1H, CH-2), 2.21 [q, J = 7.6, 2H, $-OC(O)C\mathbf{H_2}CH_3$], 2.45 (s, 3H, CH_3ArSO_2), 3.91 (dd, J = 9.5 and 6.2, 1H, CH_2-1), 4.01 (d, J = 9.5 and 4.8, 1H, CH_2-1), 4.79 (qt, J = 6.6, 1H, CH-3), 7.36 (d, J = 8.2, 2H, Ar), 7.78 (d, J = 8.3, 2H, Ar). ^13C NMR: δ 13.0, 16.9, 21.6, 27.7, 37.7, 70.8, 71.3, 127.9, 129.8, 132.8, 144.8, 173.6.

*(3*SR,*5*RS,*6*SR)- *and (3*RS,*5*RS,*6*SR)-*3,5,6-Trimethyltetrahydropyran-2H-one* ***1a/1b***. To a solution of *tert*-BuOK (0.15 mg, 1.3 mmol) in THF (10 ml) at −20°C was added dropwise a solution of **5** (0.10 g, 0.32 mmol) in THF (6.0 ml). After stirring for 20 min, the reaction was quenched with conc. HCl (0.1 ml), dried with $MgSO_4$, and the solvent was removed under reduced pressure. Purification by column chromatography (Florisil, 2% ethyl acetate in hexane) afforded a 3 : 2 mixture of lactones **1a**/**1b** (0.023 g, 0.16 mmol), in 50% yield.

IR (film): 1732 cm^{-1}. MS (*m*/*z*): 142 (3%), 127 (3%), 98 (18%), 70 (16%),

57 (13%), 56 (100%), 55 (13%). HR-MS calcd. for $C_8H_{14}O_2$: 142.0993; found: 142.0994.

1a. ^{1}H NMR: δ 0.99 (d, *J* = 6.5, 3H, CH_3-5), 1.29 (d, *J* = 7.0, 3H, CH_3-3), 1.36 (d, *J* = 6.3, 3H, CH_3-6), 1.36 (m, 1H, CH-5), 1.6–1.7 (m, 1H, CH_2-4), 1.91 (ddd, *J* = 3.1, 6.2 and 13.3, 1H, CH_2-4), 2.48–2.52 (m, 1H, CH-3), 3.89–4.20 (m, 1H, CH-6). ^{13}C NMR: δ 17.3, 17.3, 20.2, 36.0, 36.4, 37.8, 83.7, 174.6.

1b. ^{1}H NMR: δ 1.01 (d, *J* = 5.8, 3H, CH_3-5), 1.22 (d, *J* = 6.9, 3H, CH_3-3), 1.35 (d, *J* = 6.2, 3H, CH_3-6), 1.66–1.76 (m, 3H, CH_2-4 and CH-5), 2.53–2.57 (m, 1H, CH-3), 3.89–4.20 (m, 1H, CH-6). ^{13}C NMR: δ 16.5, 17.9, 19.5, 32.7, 33.5, 35.4, 80.1, 176.5.

*(2RS,3SR)-1-*O*-p-Toluenesulfonyl-3-*O*-benzyl-2-methyl-1,3-butanediol* (**7**). To a suspension of NaH (0.029 g, 1.2 mmol) in DMF (2.0 ml) at 0°C was added benzyl bromide (0.09 ml, 0.8 mmol), followed by a solution of **4** (0.10 g, 0.40 mmol) in DMF (1.5 ml). The reaction was quenched by addition of satd. NH_4Cl (4.0 ml) after 45 min at room temperature. The aqueous phase was extracted with CH_2Cl_2 (3 × 10 ml), and the organic phase washed with water (3 × 10 ml), dried over $MgSO_4$, and the solvent evaporated under reduced pressure. Purification of the crude product by column chromatography (5% ethyl acetate in hexane) afforded **7** (0.084 g, 0.24 mmol) in 60% yield as a colorless oil (found: C, 65.07; H, 7.06%; $C_{19}H_{24}O_4S$ requires C, 65.49%; H, 6.94%).

IR (film): 1360 and 1177 cm^{-1}. ^{1}H NMR: δ 0.90 (d, *J* = 7.0, 3H, CH_3-2), 1.08 (d, *J* = 6.3, 3H, CH_3-4), 1.82–1.87 (m, 1H, CH-2), 2.36 (s, 3H, CH_3ArSO_2), 3.35 (qt, *J* = 6.4, 1H, CH-3), 3.93 (dd, *J* = 4.7 and 9.5, 1H, CH_2-1), 3.94 (dd, *J* = 5.6 and 9.5, 1H, CH_2-1), 4.22 (d, *J* = 11.7, 1H, OCH_2Ar), 4.44 (d, *J* = 11.7, 1H, OCH_2Ar), 7.12–7.24 (m, 7H, Ar), 7.66 (d, *J* = 8.3, 2H, Ar). ^{13}CNMR: δ 12.8, 16.1, 21.2, 38.5, 70.2, 71.4, 74.8, 127.0, 127.2, 127.7, 127.9, 129.4, 133.9, 138.5, 143.6. MS (*m/z*): 176 (7%), 155 (7%), 108 (7%), 107 (30%), 91 (100%), 70 (26%).

E/Z-(4RS,5SR)-Ethyl-5-O-Benzyl-2,4-dimethyl-5-hydroxy-2-hexenoate (**8**). A 5 M KOH solution (9.0 ml) was added to a solution of **7** (0.49 g, 1.4 mmol) in DMSO (1.5 ml). The reaction mixture was kept at 85°C for 12 hr, and then it was extracted with ether (3 × 30 ml). The organic phase was washed with 10% HCl (2 × 20 ml) and satd. NaCl (2 × 20 ml). The organic phase was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (10% ethyl acetate in hexane) to afford (*2RS,3SR*)-3-*O*-benzyl-2-methyl-1,3-butanediol (0.16 g, 0.84 mmol) in 60% yield.

Swern oxidation (Mancuso and Swern, 1981) afforded the corresponding aldehyde (0.14 g, 0.73 mmol), in 87% yield after purification by column chromatography (2% ethyl acetate in hexane), which was dissolved in benzene (3 ml) and treated at 0°C with a benzene solution (3 ml) of the sodium salt of triethyl 2-phosphonopropionate (0.29 g, 1.1 mmol).

The reaction was stirred 1 hr, after which it was diluted with ether (5 ml) and washed with water (2 × 3 ml), 1% HCl (2 × 3.0 ml), satd. $NaHCO_3$ (2 × 3 ml), and satd. NaCl (2 × 3 ml). The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (2% ethyl acetate in hexane) to afford **8** (0.14 g, 0.51 mmol) as a 2 : 1 mixture of the *E* and *Z* isomers, in 70% yield, as a colorless oil (found: C, 74.19; H, 8.70; $C_{17}H_{24}O_3$ requires: C, 73.88; H, 8.75).

(E)-***8***. IR (film): 1711 cm^{-1}. ^1H NMR (CCl_4): δ 1.02 (d, J = 6.9, 3H, CH_3-4), 1.12 (d, J = 6.2, 3H, CH_3-6), 1.30[t, J = 7.1, 3H, $C(O)OCH_2C\mathbf{H_3}$], 1.79 (d, J = 1.4, 3H, CH_3-2), 2.59–2.68 (m, 1H, CH-4), 3.37 (qt, J = 6.2, 1H, CH-5), 4.14 [q, J = 7.1, 2H, $C(O)OC\mathbf{H_2}CH_3$], 4.39 (d, J = 12.1, 1H, OCH_2Ph), 4.55 (d, J = 12.1, 1H, OCH_2Ph), 6.54 (dq, J = 1.4 and 10.0, 1H, CH-3), 7.10–7.30 (m, 5H, Ar). ^{13}C NMR (CCl_4): δ 12.4, 14.3, 15.3, 16.4, 38.5, 59.5, 70.2, 76.9, 126.0, 127.1, 127.6, 127.8, 139.0, 143.1, 166.3. MS (*m/z*): 232 (5%), 141 (14%), 135 (10%), 113 (33%), 91 (100%).

(Z)-***8***. ^1H NMR (CCl_4): δ 1.01 (d, J = 6.5, 3H, CH_3-4), 1.07 (d, J = 6.2, 3H, CH_3-6), 1.29 [t, J = 7.1, 3H, $C(O)OCH_2C\mathbf{H_3}$], 1.88 (d, J = 1.4, 3H, CH_3-2), 3.30–3.50 (m, 2H, CH-4 and CH-5), 4.14 [q, J = 7.1, 2H, $C(O)OC\mathbf{H_2}CH_3$], 4.40 (d, J = 12.1, 1H, OCH_2Ph), 4.54 (d, J = 12.0, 1H, OCH_2Ph), 5.80 (dq, J = 1.4 and 9.6, 1H, CH-3), 7.10–7.30 (m, 5H, Ar). ^{13}C NMR (CCl_4): δ 14.2, 16.1, 16.7, 20.7, 37.7, 59.2, 70.0, 77.4, 126.8, 127.1, 127.6, 127.7, 138.6, 144.6, 166.2. MS (*m/z*): 232 (5%), 141 (14%), 135 (10%), 113 (33%), 91 (100%).

*(3*SR,*5*RS,*6*SR*)- and (3*RS,*5*RS,*6*SR*)-3,5,6-Trimethyltetrahydropyran-2H-one* (***1a/1b***). A suspension of a catalytic amount of 10% Pd/C in a solution of ester **8** (0.41 g, 1.5 mmol) in ethanol (8.0 ml) was stirred 48 hr under hydrogen (3 atm) at room temperature. The mixture was filtered on Celite, the solvent removed under reduced pressure, the crude product diluted with benzene (2.0 ml), and a catalytic amount of *p*-toluenesulfonic acid (10 mg) was added. After stirring 12 hr at room temperature, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (Florisil, 2% ethyl acetate in hexane) to afford a 2 : 1 mixture of lactones **1a/1b** (0.13 g, 0.90 mmol) in 60% yield.

*(4*RS,*5*SR*)-5-Hydroxy-2,4-dimethyl-2-*O*-trimethylsilyl-3-hexanone* (***10***). To a solution of diisopropylamine (0.88 ml, 6.3 mmol) in THF (10.5 ml) at −78°C was added a 2.5 M solution of *n*-BuLi (2.3 ml, 5.7 mmol). After stirring 15 min, a solution of ketone **9** (1.0 g, 5.3 mmol) in THF (2.0 ml) was added dropwise and the reaction was stirred for 1 hr at −78°C; then a solution of acetaldehyde (0.70 g, 16 mmol) in THF (2.0 ml) was added. After 45 min at −78°C, the reaction was quenched by addition of satd. NH_4Cl (2.5 ml) and the mixture was warmed to room temperature. The reaction mixture was diluted with ether (10 ml) and the organic phase was washed with 1% HCl (2 × 5 ml), satd. $NaHCO_3$ (5 ml), and satd. NaCl (5 ml). The organic phase was dried over $MgSO_4$ and the

solvent was removed under reduced pressure. The crude product was purified by Kugelrohr destillation (2 mm Hg, 65–70°C) to afford **10** (1.11 g, 4.77 mmol) as colorless oil in 90% yield.

IR (film): 3432 and 1709 cm^{-1}. ^1H NMR (CCl_4): δ 0.19 [s, 9H, $OSi(CH_3)_3$], 1.08 (d, J = 6.4, 3H, CH_3-6), 1.09 (d, J = 7.0, 3H, CH_3-4), 1.33 (s, 3H, CH_3-1), 1.36 (s, 3H, CH_3-2), 2.69–2.85 (s, br, 1H, OH), 3.18 (dq, J = 3.7 and 7.0, 1H, CH-4), 3.89 (dq, J = 3.7 and 6.4, 1H, CH-5). ^{13}C NMR (CCl_4): δ 2.2, 11.0, 20.0, 27.0, 27.3, 44.4, 67.3, 80.1, 218.0. MS (m/z): 132 (12%), 131 (100%), 75 (26%), 73 (75%), 45 (12%).

*(4*RS,*5*SR*)-5-O-Acetyl-2,4-dimethyl-2,5-dihydroxy-3-hexanone* (***11***). To a solution of **10** (1.73 g, 7.45 mmol) in CH_2Cl_2 (15 ml) were added Et_3N (1.24 ml, 8.93 mmol), acetic anhydride (0.84 ml, 8.9 mmol), and a catalytic amount of 4-*N*,*N*-dimethylaminopyridine. The reaction mixture was stirred for 30 min at room temperature, diluted with CH_2Cl_2 (10 ml), and the organic phase was washed with water (2 × 5 ml), 5% HCl (2 × 5 ml), satd. $NaHCO_3$ (2 × 5 ml), and satd. NaCl (2 × 5 ml). The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by Kugelrohr distillation (2 mm Hg, 85–90°C) and the *O*-acetyl derivative (1.73 g, 6.33 mmol, 85% yield) was dissolved in THF (13 ml) and a solution of acetic acid (3.7 ml) in water (12.5 ml). After 14 hr at room temperature, the reaction mixture was extracted with ether (2 × 25 ml) and washed with satd. $NaHCO_3$ (3 × 20 ml) and satd. NaCl (2 × 15 ml). The organic phase was dried over $MgSO_4$, the organic solvent removed under reduced pressure, and the crude product was purified by column chromatography (20% ethyl acetate in hexane) to afford **11** (1.02 g, 5.06 mmol) as a colorless oil in 80% yield.

IR (film): 3485, 1734 and 1711 cm^{-1}. ^1H NMR: δ 1.15 (d, J = 6.9, 3H, CH_3-4), 1.22 (d, J = 6.5, 3H, CH_3-6), 1.38 (s, 3H, CH_3-1), 1.40 (s, 3H, CH_3-2), 2.05 [s, 3H, $OC(O)CH_3$], 3.27 (qt, J = 6.9, 1H, CH-4), 3.61–3.69 (s, br, 1H, OH), 5.17 (qt, J = 6.5, 1H, CH-5). ^{13}C NMR: δ 14.6, 17.8, 21.2, 26.3, 26.5, 43.9, 72.0, 77.2, 170.7, 216.1. MS (m/z): 119 (16%), 83 (11%), 59 (100%), 56 (49%), 43 (55%).

*(E/Z)-(4*SR,*5*SR*)-Ethyl-5-O-acetyl-2,4-dimethyl-5-hydroxy-2-hexenoate* (***12***). To a solution of **11** (0.98 g, 4.85 mmol) in methanol (10 ml) at 0°C was added $NaBH_4$ (0.37 g, 9.7 mmol) portionwise. The reaction was stirred 1 hr at 0°C and acidified with 5% HCl (pH 4–5). The reaction mixture was saturated with solid NaCl and extracted with ether (3 × 15 ml). The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was dissolved in ethanol (30 ml) and aqueous $NaIO_4$ solution (0.20 M, 60 ml) was added dropwise at 0°C, followed by aqueous NaOH (0.25 M, 2.0 ml). The reaction was kept 1 hr at 0°C, poured in water (20 ml) and extracted with CH_2Cl_2 (3 × 10 ml). The organic phase was washed with water (3 × 10 ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure to furnish the (2*RS*,3*SR*)-3-*O*-acetyl-

2-methyl-2-hydroxybutyraldehyde (0.49 g, 3.4 mmol) in 70% combined yield (2 steps), which was used without further purification.

The aldehyde was dissolved in benzene (3.3 ml) and treated at 0°C with a benzene solution (4.4 ml) of the sodium salt of triethyl 2-phosphonopropionate (1.34 g, 5.16 mmol). The reaction was stirred 1 hr at 0°C, diluted with ether (20 ml), and washed with water (2 × 10 ml), 1% HCl (2 × 5 ml), satd. $NaHCO_3$ (2 × 5 ml), and satd. NaCl (2 × 5 ml). The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (2% ethyl acetate in hexane) to afford **12** (0.39 g, 1.7 mmol) in 50% yield as a 1.2 : 1 mixture of the *Z* and *E* isomers, and **13** (0.23 g, 1.4 mmol) in 40% mmol) in 40% yield (found: C, 63.34; H, 9.02; $C_{12}H_{20}O_4$ requires, C, 63.14; H, 8.83).

IR (film): 1735 and 1711 cm^{-1}. MS (*m/z*): 184 (7%), 142 (100%), 141 (19%), 114 (30%), 43 (30%).

(Z)-***12***. ^1H NMR (CCl_4): δ 0.97 (d, *J* = 6.8, 3H, CH_3-4), 1.114 (d, *J* = 6.3, 3H, CH_3-6), 1.30 (t, *J* = 7.1, 3H, $OCH_2\mathbf{CH_3}$), 1.89 (d, *J* = 1.5, 3H, CH_3-2), 1.97 [s, 3H, $OC(O)CH_3$], 3.21–3.41 (m, 1H, CH-4), 4.16 (q, *J* = 7.1, 2H, $\mathbf{OCH_2}CH_3$), 4.68 (qt, *J* = 6.3, 1H, CH-5), 5.63 (dq, *J* = 1.5 and 10.2, 1H, CH-3). ^{13}-C NMR (CCl_4): δ 12.6, 14.2, 15.9, 17.5, 20.9, 38.0, 59.8, 73.3, 128.0, 142.6, 167.1, 169.5.

(E)-***12***. ^1H NMR (CCl_4): δ 1.02 (d, *J* = 6.8, 3H, CH_3-4), 1.16 (d, *J* = 6.3, 3H, CH_3-6), 1.29 (t, *J* = 7.1, 3H, $OCH_2\mathbf{CH_3}$), 1.82 (d, *J* = 1.5, 3H, CH_3-2), 1.98 [s, 3H, $OC(O)CH_3$], 2.26–2.28 (m, 1H, CH-4), 4.14 (q, *J* = 7.1, 2H, $\mathbf{OCH_2}CH_3$), 4.73 (qt, *J* = 6.3, 1H, CH-5), 6.47 (dq, *J* = 1.5 and 10.3, 1H, CH-3). ^{13}C NMR (CCl_4): δ 12.6, 14.2, 16.1, 17.3, 20.9, 37.7, 60.2, 73.3, 128.7, 141.8, 167.2, 169.7.

13. ^1H NMR (CCl_4): δ 1.28 (t, *J* = 7.1, 3H, $OCH_2\mathbf{CH_3}$), 1.74 (d, *J* = 6.8, 3H, CH_3-6), 1.83 (s, 3H, CH_3-4), 1.94 (s, 3H, CH_3-2), 4.14 (q, *J* = 7.1, 2H, $\mathbf{OCH_2}CH_3$), 5.64 (q, *J* = 6.8, 1H, CH-5), 6.99 (s, 1H, CH-3). ^{13}C NMR (CCl_4): δ 13.8, 13.9, 14.3, 15.9, 59.7, 125.3, 129.4, 133.1, 142.1, 167.5.

*(3*SR,*5*RS,*6*RS*)- and (3*RS,*5*RS,*6*RS*)-3,5,6-Trimethyltetrahydropyran-2H-one* (***1c*** and ***1d***). A suspension of a catalytic amount of 10% Pd/C in a solution of **12** (0.18 g, 0.79 mmol) in ethanol (40 ml) was stirred 17 hr under a hydrogen atmosphere (3 atm). The mixture was filtered on Celite and the solvent was removed under vacuum. The residue was dissolved in methanolic KOH (1.0 M, 4.0 ml), and the reaction mixture was refluxed for 2 hr. The solvent was removed under reduced pressure, the residue was treated with 5% HCl until acidic (pH 3–4), and the aqueous phase was saturated with solid NaCl and extracted with ether (3 × 10 ml). The solvent was removed under reduced pressure and the residue was dissolved in benzene (2.0 ml) and treated overnight with a catalytic amount of *p*-toluenesulfonic acid. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (Florisil, 2% ethyl acetate in hexane) to afford a 4 : 1 mixture of **1d**/**1c** (0.067 g, 0.47 mmol) in 60% yield.

IR (film): 1734 cm^{-1}. MS (*m/z*): 142 (2%), 127 (3%), 98 (11%), 70 (16%), 57 (11%), 56 (100%), 55 (20%). HR-MS calcd. for $C_8H_{14}O_2$: 142.0993; found: 142.0994.

1c. ^1H NMR (CCl_4): δ 0.94 (d, *J* = 7.0, 3H, CH_3-5), 1.14 (d, *J* = 6.7, 3H, CH_3-3), 1.27 (d, *J* = 6.6, 3H, CH_3-6), 1.62–1.72 (m, 1H, CH-5), 2.02–2.24 (m, 2H, CH_2-4), 2.43–2.49 (m, 1H, CH-3), 4.43 (dq, *J* = 6.6 and 3.3, 1H, CH-6). ^{13}C NMR (CCl_4): δ 15.4, 16.3, 16.7, 31.1, 32.9, 34.6, 75.3, 171.2.

1d. ^1H NMR (CCl_4): δ 1.01 (d, *J* = 7.0, 3H, CH_3-5), 1.23 (d, *J* = 7.1, 3H, CH_3-3), 1.27 (d, *J* = 6.6, 3H, CH_3-6), 1.62–1.72 (m, 1H, CH-5), 1.84–1.98 (m, 2H, CH_2-4), 2.43–2.49 (m, 1H, CH-3), 4.44 (dq, *J* = 6.7 and 3.3, 1H, CH-6). ^{13}C NMR (CCl_4): δ 11.8, 17.9, 18.0, 30.7, 30.8, 35.8, 78.3, 171.2.

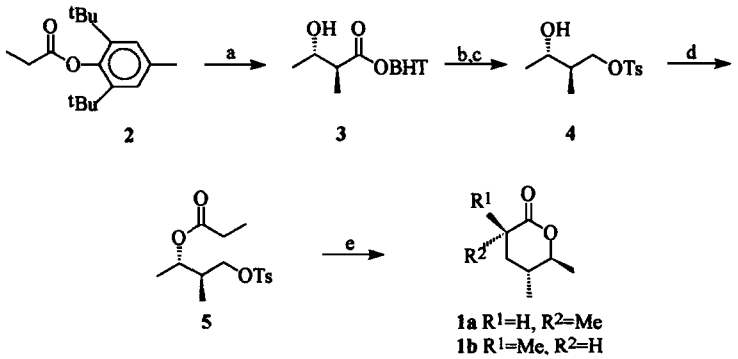
RESULTS AND DISCUSSION

Based on our previous experience on the total synthesis of ant pheromones (Pilli and Murta, 1993), we decided to approach the determination of the relative configuration of the trisubstituted tetrahydropyran-2H-one isolated from male *Calomyrmex* sp. ants by chemical synthesis of authentic samples of δ-lactones **1a/1b** and **1c/1d**. We expected that GC analyses on apolar (100% dimethylpolysiloxane) and polar (polyethylene glycol) stationary phases would allow us to determine the relative configuration of the natural lactone, since it has been previously reported (Brown and Moore, 1979) that the first eluting isomer in the synthetic mixture of the four possible racemates matched the retention time, melting point, and mass spectrum of the natural product.

Among the methodologies available for the construction of the tetrahydropyran-2H-one system, the utilization of an intramolecular alkylation (Nakai et al., 1985; Mori et al., 1991; White et al., 1992) seemed to be particularly attractive as the relative configuration of the stereogenic centers at C-5 and C-6 (Scheme 1) could be unambiguously established by stereoselective aldol condensation (Heathcock, 1993), and the stereogenic center at C-3 could be established during the intramolecular alkylation step. We previously used this plan during the total synthesis of (±)-invictolide (Pilli and Murta, 1993), a constituent of the queen recognition pheromone of the fire ant *Solenopsis invicta* and (−)-serricornine, the sex pheromone of the cigarette beetle *Lasioderma serricorne* (Pilli and de Andrade, 1994).

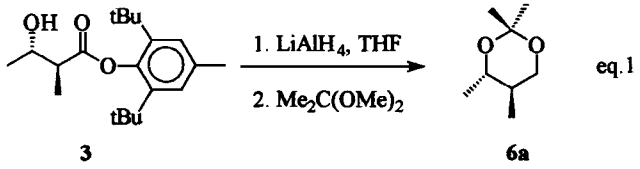
The *trans* relationship at C-5/C-6 of **1a** and **1b** was secured through the stereoselective aldol condensation of the *E*-lithium enolate derived from 2,6-di-*tert*-butyl-4-methylphenyl propionate **2** (Heathcock *et al.*, 1981) and acetaldehyde (Scheme 1).

The *anti*-hydroxy ester **3** was obtained in 60% yield as a colorless solid (mp 109.9–110.7°C) after recrystallization from hexane. The *anti* configuration of hydroxy ester **3** was unambiguously established after its conversion to acetonide



SCHEME 1. (a) i. LDA, THF, −78°C; ii. MeCHO (60%); (b) $LiAlH_4$, THF, rt. (70%); (c) TsCl, Et_3N, CH_2Cl_2, DMAP(cat.), −15°C (90%); (d) $(EtCO)_2O$, Et_3N, CH_2Cl_2, rt. (85%); (e) *t*-BuOK, THF, −20° to 0°C (50%).

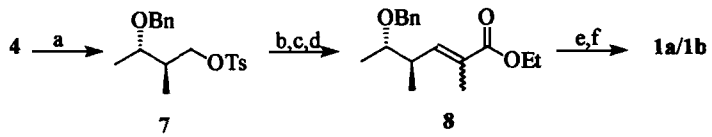
6a (equation 1) and analysis of its ^1H NMR spectrum (Lipshutz et al., 1988; Rychnovsky et al., 1993).



H-5 appeared as a multiplet at δ1.40–1.52 ppm and H-4 as a double quartet at δ3.46 ppm (J = 6.1 and 10.0 Hz), which, upon irradiation of the methyl group at C-4, collapsed to a doublet (J = 9.2 Hz), as expected for the *trans* relationship between H-4 and H-5.

Attempts to carry out the intramolecular alkylation of **5** with lithium or potassium diisopropylamide either in THF or ether did not yield lactones **1a/1b**. However, the reaction proceeded smoothly when a solution of **5** in THF was added dropwise to a THF solution of 4.0 equivalents of freshly sublimed potassium *tert*-butoxide to afford a 3 : 2 mixture of lactones **1a/1b** in 50% yield.

The configuration of the stereogenic center created during the intramolecular alkylation was established through analysis of the ^1H and ^{13}C NMR spectra of the mixture: H-3 in the major isomer appeared at higher field (multiplet, δ2.46–2.58 ppm) than in the minor isomer (multiplet, δ2.58–2.72 ppm), which was the same trend observed for (±)-invictolide and its C-3 epimer (Pilli and Murta, 1993). In the ^{13}C NMR spectra, C-2 in the major isomer and C-6 in the



SCHEME 2. (a) BnBr, NaH, DMF, rt. (60%); (b) KOH, H_2O, DMSO, 80–90°C (60%); (c) $(COCl)_2$, DMSO, CH_2Cl_2, −78°C; then Et_3N, −78°C to rt. (87%); (d) $(EtO)_2P(O)CH(Me)CO_2Et$, NaH, C_6H_6, 0°C (70%); (e) H_2, Pd/C, EtOH; (f) *p*-TsOH, C_6H_6, rt. (60%, two steps).

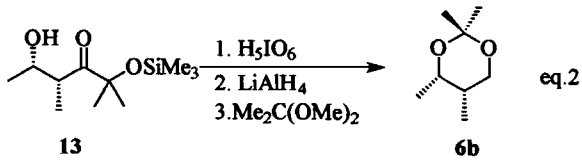
minor one appeared more shielded (δ174.6 and 80.90 ppm, respectively) than the corresponding carbons of their C-3 epimers (δ176.5 and 83.7 ppm, respectively), the same behavior previously observed for (±)-invictolide and its C-3 epimer (Pilli and Murta, 1993).

Despite the conciseness of the route described above for lactones **1a/1b**, the key step in such an approach (intramolecular alkylation) failed to provide reproducible yields. In many instances, lactones **1a/1b** were isolated in disappointingly low yields (ca. 10%). Although the route described in Scheme 1 did provide us lactones **1a/1b** for further comparisons, we decided to pursue an alternative procedure to prepare lactones **1a/1b** and **1c/1d**.

The alternative route (Scheme 2) was based on the chain elongation of protected diol **7** via its conversion to aldehyde and Wittig-Horner homologation via the sodium salt of triethyl 2-phosphonopropionate. A 2 : 1 mixture of unsaturated esters **8** favoring the *E* isomer was obtained, converted to the corresponding saturated hydroxy ester and lactonized to afford a 2 : 1 mixture of lactones **1a/1b**.

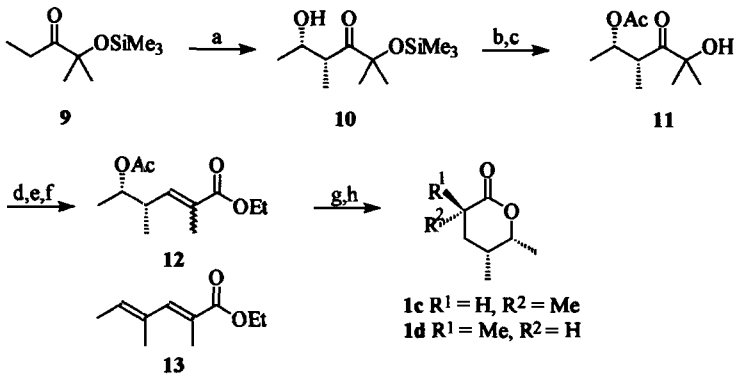
Similarly, a mixture of δ-lactones **1c/1d** (Scheme 3) was prepared starting with the stereoselective aldol condensation of acetaldehyde and the lithium enolate of the *syn*-selective silyloxyketone **9** (Heathcock et al., 1980).

The *syn*-hydroxy ketone **10** was obtained in 90% yield as a colorless oil. The *syn* stereochemistry was established after its conversion to acetonide **6b** (equation 2) and analysis of its ^{1}H-NMR spectrum (Lipshutz et al., 1988; Rychnovsky et al., 1993).



eq.2

The carbinolic proton (H-4) appeared as a double quartet (J = 6.4 and 2.6 Hz)



SCHEME 3. (a) i. LDA, THF, −78°C; ii. MeCHO (90%); (b) Ac_2O, Et_3N, DMAP(cat), CH_2Cl_2 (85%); (c) aq. HOAc, THF (80%); (d) $NaBH_4$, MeOH, 0°C (90%); (e) $NaIO_4$, H_2O, EtOH, 0°C (78%); (f) $(EtO)_2P(O)CH(Me)CO_2Et$, NaH, C_6H_6, 0°C (**12**, 50%; **13**, 40%); (g) H_2, Pd/C, EtOH (95%); (h) i. KOH, MeOH, reflux; ii. *p*-TsOH, C_6H_6, rt. (60%).

at δ 4.04 ppm and a collapsed to a doublet (J = 2.2 Hz) upon irradiation of the methyl group at C-4.

Hydroxy ketone **10** was converted to unsaturated esters **12** (68% yield), which yielded a 4 : 1 mixture of δ-lactones **1d/1c** (Scheme 3).

Analogously to the pattern described for (−)-serricornine (Redlich et al., 1988), in the ^{1}H NMR spectrum of the mixture, the methyl group at C-3 appeared downfield in the major isomer (δ 1.23 ppm) when compared with the corresponding protons in the minor one (δ 1.14 ppm).

GC-MS analysis (Carbowax 20 M and HP-1 columns, 25 m × 0.20 mm ID, film thickness 0.33 μm) of the synthetic lactones **1a/1b** and **1c/1d** revealed the same fragmentation pattern for lactones **1a**, **1b** and **1c**, **1d** as described by Brown and Moore (1979) for the natural lactone including the molecular ion at m/z 142, the characteristic loss of CO_2 (m/z 98), and the base peak at m/z 56, which corresponded to the loss of methylketene. Moreover, lactone **1a** was the first eluting component of the synthetic mixture of lactones (retention time: 11.87, 12.44, 13.18, and 13.46 min. for lactones **1a**, **1b**, **1d**, and **1c**, respectively) as observed by Brown and Moore (1979) when the natural lactone was compared with a synthetic mixture of the four stereoisomers (Figure 2).

Based on the results described above, the structure of the δ-lactone present in the mandibular gland secretion of *Calomyrmex* sp. males and described by Brown and Moore (1979) as 2,4-dimethylhexan-5-olide was assigned as (3*SR*,5*RS*,6*SR*)-3,5,6-trimethyltetrahydropyran-2H-one (**1a**).

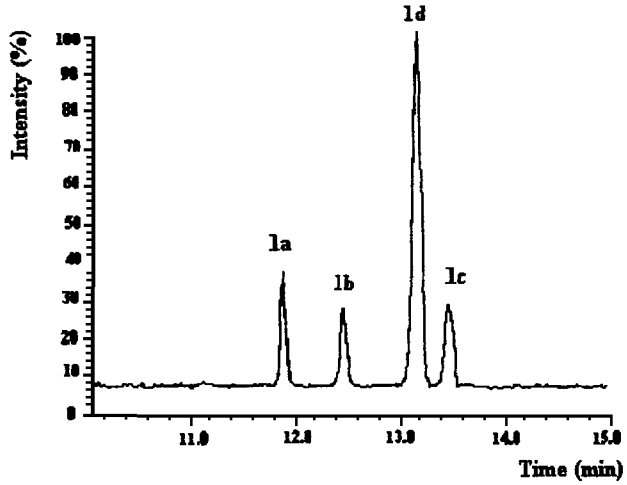


FIG. 2. GC analysis of the synthetic mixture of lactones **1a**, **1b**, **1c**, and **1d**.

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EFFECT OF *Solanum* GLYCOSIDES ON THE APHID *Schizaphis graminum*

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Abstract—Common *Solanum* glycosides were tested against a non-*Solanum* pest, the greenbug, *Schizaphis graminum*, fed by artificial diet supplemented with the tested compounds. In contrast with the potato aphid *Macrosiphum euphorbiae*, *S. graminum* is sensitive to both potato and tomato glycoalkaloids, as well as to all the different *Solanum* steroidal glycosides tested. *S. graminum* was less sensitive to glycoalkaloids than to the steroidal glycosides. Laxumin A (LC_{50} 4.3 μM) and laxumin B (LC_{50} 6.1 μM), isolated from *Solanum laxum*, were the most active of the tested compounds. The different sensitivity of both aphids against *Solanum* glycosides could be related to a possible adaptation of the insects to their host plants secondary metabolites.

Key Words—*Schizaphis graminum*, Aphididae, aphids, greenbug, survival, glycosides, glycoalkaloids.

INTRODUCTION

Plant secondary metabolites can act as protective agents in plants against insects either by repellence or by direct toxicity (Montllor, 1991; Janzen et al., 1977). Many different types of secondary metabolites including alkaloids, terpenes, steroids, iridoid glycosides, aliphatic molecules, phenolics, and others, have been demonstrated to confer resistance to different plant species against pathogens. Among them, steroidal glycosides and glycoalkaloids are well-known resistance

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factors in *Solanum* plants, providing them with protection against many insect pests, in particular the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and the potato leafhopper, *Empoasca fabae* (Harris) (Tingey, 1984; Tingey et al., 1978), two important potato pests.

In previous work (Güntner et al., 1997) we tested some *Solanum* glycoalkaloids against the potato aphid, *Macrosiphum euphorbiae* (Thomas). This insect is the most important potato virus vector and is responsible for important losses in potato tuber production (Raman and Radcliffe, 1992). The data obtained indicated that glycoalkaloids, although with some differences in activity, did not strongly affect *M. euphorbiae* vital parameters. This is in agreement with the hypothesis that the aphid should be tolerant to the host-plant secondary metabolites, particularly the glycoalkaloids. In order to further challenge the idea that the aphid is adapted to its host-plant allelochemicals, we tested *Solanum* glycosides against an aphid, the greenbug, *Schizaphis gramunum* (Rondani), which does not normally settle on *Solanum* plants. This aphid is also of economic interest, since it is considered the worst cereal aphid pest (Anonymous, 1981).

In this paper we report the toxic effect of several *Solanum* steroidal glycosides and glycoalkaloids on the greenbug aphid, the influence of some of the structural features of the glycosides on activity, and the possible implications of the role of these compounds on insect feeding preferences.

METHODS AND MATERIALS

Compounds Tested. Solanine, chaconine, tomatine, and tomatidine were purchased from Sigma, St Louis, Missouri Laxumin A and B, foliumin A, and foliumin were obtained as in Ferreira et al. (1994, 1996).

Aphids. Schizaphis graminum (Rondani) were collected from colonies reared on *Hordeum distichum* cv. Aramir grown under a photoperiod of 12 hr light in the laboratory at 25 ± 4°C.

Assay. Aphid toxicity assays were conducted with a holidic diet (Argandoña et al., 1982) placed between two layers of parafilm M to make a sachet. The compounds were added to the diet at varying concentrations. Controls were sachets filled only with diet. Nonchoice assays were performed under ambient light and the mortality of aphids was determined at 24 hr. Five replicates containing 10 aphids each were used for each treatment.

Data Analysis. The percentage of survival is calculated as follows:

$$100 * (100-; \text{mortality})\ \text{treatment}/(100-; \text{mortality})\ \text{control}.$$

Statistical treatment to detect significant differences between control and test substances was done by the Student's t test for $\alpha = 0.05$ (Snedecor and Cochran,

1977). LC_{50} values were calculated by probit analysis according to Finney (1971).

RESULTS AND DISCUSSION

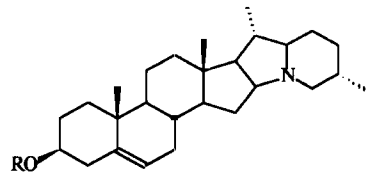
Two types of *Solanum* secondary metabolites—glycoalkaloids and steroidal glycosides (Figure 1)—were tested against *S. graminum*. These compounds are widely distributed in *Solanum* species and are also common in other *Solanaceae* genera. Solanine (**2**), chaconine (**3**), and tomatine (**5**) were the three glycoalkaloids assayed. The former two are the most common potato glycoalkaloids, and they are present in different amounts in the whole plant. Their concentration depends on the plant part, the cultivar variety, the plant developmental stage, and environmental and cultivation conditions (Friedman and McDonald, 1997). Both glycoalkaloids share the same aglycone, solanidine (**1**), linked to a trisaccharide residue, solatrios (**2**) and chacotriose (**3**). Tomatine, present in tomato (*Lycopersicon esculentum* L.), but not in potato, and its aglycone, tomatidine (**4**), were also assayed.

The steroidal glycosides tested were isolated from Uruguayan wild *Solanum* species. Foliumin (**6**) and foliumin A (**7**), obtained from *S. amygdalifolium* (Ferreira et al., 1994) comprise a spirosten δ-lactone aglycone linked to a β_2-chacotriose (**7**) or chacotriose (**6**) residue. Laxumins A (**8**) and B (**9**) are composed of spirosten aglycone linked to tetra- (e) or trisaccharide (f) residues, respectively (Figure 1) (Ferreira et al., 1996).

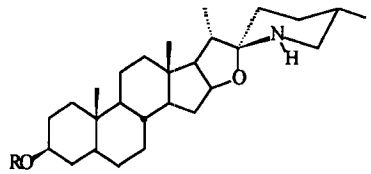
The biological activity of the compounds was measured by feeding *S. gramium* on an artificial diet supplemented with each compound to be tested. The relation between allelochemical concentration in the diet and the survival of *S. graminum* is shown in Table 1.

With respect to activity against *S. graminum*, three groups of compounds can be distinguished. The first group consists of tomatidine, which was the only inactive compound ($LC_{50} > 5000$ μM). A second group with intermediate activity included the potato glycoalkaloids, solanine (LC_{50} 138 μM) and chaconine (LC_{50} 137 μM), and foliumin A (LC_{50} 137 μM). A third group comprising very active compounds included tomatine (LC_{50}, 7.3 μM), foliumin (LC_{50} 19.7 μM), laxumin A (LC_{50} 4.3 μM), and laxumin B (LC_{50} 6.1 μM).

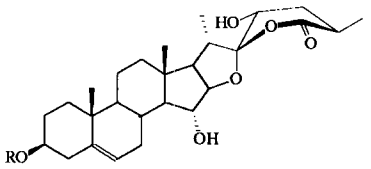
Several postulates can be made in relation to the structure of the tested *Solanum* glycosides and their activity. In the first place, the marked difference in activity between tomatine and its aglycone tomatidine highlights the profound effect of the oligosaccharide residue on glycoalkaloid biological activity, in agreement with previous results (Friedman and McDonald, 1997). The carbohydrate moiety influences the activity in a subtle way, and not only the presence or absence of the oligosaccharide residue affects activity, but slight modifications



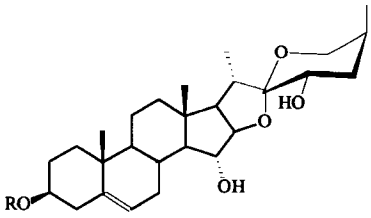
R substituent	Compound	
H	solanidine	1
solatriosyl	solanine	2
chacotriosyl	chaconine	3



R substituent	Compound	
H	tomatidine	4
lycotetraose	tomatine	5



R substituent	Compound	
chacotriose	foliumin	6
β2-chacotriose	foliumin A	7



R substituent	Compound	
e	laxumin A	8
f	laxumin B	9

oligosaccharide	oligosaccharide residue structure
solatriose	β-D-Glc*p*-(1→3)-[α-L-Rha*p*-(1→2)]-β-D-galactopyranoside
chacotriose	α-L-Rha*p*-(1→4)-[α-L-Rha*p*-(1→2)]-β-D-glucopyranoside
lycotetraose	β-D-Glc*p*-(1→2)-[β-D-Xyl*p*-(1→3)]-β-D-Glc*p*-(1→4)-β-D-galactopyranoside
β_2-chacotriose	α-L-Rha*p*-(1→2)-β-D-glucopyranoside
e	β-D-Glc*p*-(1→2)-β-D- Glc*p*-(1→4)-[α-L-Rha*p*-(1→2)]-β-D-galactopyranoside
f	β-D-Glc*p*-(1→4)-[α-L-Rha*p*-(1→2)]-β-D-galactopyranoside

FIG. 1. Chemical structures of the tested compounds.

in the carbohydrate moiety can have a marked effect on it. This can be observed in the different activity of foliumin and foliumin A. The compounds differ only in the structure of the oligosaccharide residue attached to the spirostenic aglycone. While the former contains a disaccharide, the second, which is 10 times

TABLE 1. EFFECT OF *Solanum* GLYCOSIDES ON *Schizaphis graminum* (RONDANI) EXPRESSED AS PERCENTAGE SURVIVAL AFTER FEEDING APHIDS 24 HR ON ARTIFICIAL DIET CONTAINING TEST COMPOUNDS[a]

	Concentration (μM)				
	0	50	100	250	500
Tomatidine	100 ± 7	100 ± 8	100 ± 4	93 ± 16	90 ± 12
Chaconine	100 ± 7	79 ± 8	62 ± 6	52 ± 6	NA*
Solanine	100 ± 17	68 ± 8	64 ± 8	39 ± 4	18 ± 4
Foliumin A	100 ± 8	75 ± 5	50 ± 4	50 ± 4	13 ± 2
Tomatine	100 ± 10	30 ± 5	15 ± 4	15 ± 4	25 ± 4
Foliumin	100 ± 11	38 ± 5	24 ± 6	14 ± 6	12 ± 3
Laxumin A	100 ± 8	17 ± 2	11 ± 2	17 ± 6	11 ± 2
Laxumin B	100 ± 11	24 ± 4	40 ± 8	5 ± 2	19 ± 4

[a]NA, data not available.

less toxic against the aphid, has a trisaccharide derived from the disaccharide by addition of an extra rhamnopyranosyl unit on position 4 of the glucopyranosyl residue.

Solanine and chaconine showed similar toxicity even though they have different trisaccharide moieties linked to the same aglycone.

Although tomatine was a very active compound, the glycoalkaloids as a group were less active than the oxygenated steroidal glycosides. Moreover, survival of only 17% and 24% of the aphids treated with laxumin A and laxumin B, respectively, at concentrations as low as 50 μM was strongly affected by the steriodal glycosides.

To get an insight into the toxicity of both laxumins against *S. graminum*, we can compare their LC_{50} of $\leq 6\mu$M with the corresponding LC_{50} value of 1500 μM for DIMBOA, based on data from Argandoña et al. (1981). The latter is a toxic alkaloid present in cereals and reputed to be able to confer resistance against *S. graminum*.

In a previous work (Güntner et al., 1997) we tested the activity of the glycoalkaloids used in this work against the potato aphid, *M. euphorbiae*. Although the compounds showed differential activity, as a group they had relatively low activity. These glycoalkaloids are present in its usual host plant and most probably in the aphids' diet, and these results suggest a coevolutionary adaptation between host and herbivore. In contrast, *S. graminum*, which normally feeds on Graminae, is not adapted and was significantly affected by these compounds.

The differential effects of the tested glycoalkaloids on the two aphids with different host-plant preferences are in agreement with the hypothesis of an adaptative tolerance to the secondary metabolites of their respective host plants.

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GUSTATORY RESPONSES OF GHOST CRAB *Ocypode quadrata* TO SEAWATER EXTRACTS AND CHEMICAL FRACTIONS OF NATURAL STIMULI

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Abstract—The efficacy of seawater-extracted fresh and decomposing blue crab (*Callinectes sapidus*) claw muscle homogenates as stimulants of feeding behavior by the ghost crab (*Ocypode quadrata*) was tested with cheliped flexion as a bioassay. Stimulatory components of extracts were heat-stable and <1 kDa. Fresh seawater extracts of muscle tissue homogenate elicited the most responses and decreased in efficacy with decomposition. Ultrafiltrates <1 kDa also became less stimulating with increasing decay of the homogenate. When ultrafiltrates were extracted with ethyl ether, the aqueous phase elicited the most responses. To some degree, active components were soluble in ether. Ion-exchange chromatography of the aqueous phase yielded eluates containing neutral and acidic compounds, which, following a peak in activity, became less stimulatory over time. In contrast, eluates containing amphoteric and basic compounds remained highly effective throughout bacterial degradation. However, their activity was significantly suppressed when they were mixed with neutral and acidic compounds isolated from the same samples. Mixture suppression may function as a mechanism ensuring the consumption of high-quality foods. The ability of *O. quadrata* to respond to both fresh and decomposing tissues contributes to this species' flexibility in foraging strategies and its success as an inhabitant of sandy beaches.

Key Words—Chemoreception, crustacean, decomposition, ecology, feeding behavior, food extracts, ghost crab, gustation, *Ocypode quadrata*, sensory ecology.

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INTRODUCTION

The ability of an organism to distinguish the composite chemical compounds and metabolites of prey and the subsequent integration of this information determines the type of feeding behavior exhibited during food acquisition. A correlation between chemical feeding stimulants and diet has been demonstrated for several crustaceans when single pure compounds, mixtures, and food extracts were tested (Ache, 1977). For crustaceans, chemoreception is a primary sensory modality for the detection of food (Bardach, 1975; Atema, 1977; Ache, 1982).

The western Atlantic ghost crab *Ocypode quadrata* Fabricius responds to a wide assortment of low-molecular-weight compounds when applied to contact chemoreceptors located on the chelipeds and dactyls (Trott and Robertson, 1984). These compounds are components of either fresh or decomposing food. Since crustacean chemoreceptors responded differently to stimulatory compounds when tested as mixtures rather than in pure chemical solutions (Carr and Derby, 1986), relationships between chemostimulants and diet might be better explored by testing natural mixtures, i.e., food extracts.

Flexibility in feeding behavior is characteristic of crabs in the genus *Ocypode*. For example, in addition to deposit feeding (Robertson and Pfeiffer, 1982), the semiterrestrial ghost crab *Ocypode quadrata* has been described as a predator (Cowles, 1908; Boone, 1934; Phillips, 1940; Milne and Milne, 1946; Loesch, 1957; Wade, 1967; Fales, 1976; Wolcott, 1978) and a scavenger (Cowles, 1908; Pearse, 1929; Boone, 1934; Wolcott, 1978). Which feeding behavior predominates appears to be influenced by the abundance and type of food available at study sites.

This study was designed to determine if *Ocypode quadrata* responds differently to seawater extracts of freshly injured and decomposing tissues to evaluate the possible priority of such foods in the diet of this species. In addition, the stimulatory components of the test extracts were partially characterized.

METHODS AND MATERIALS

Collection and Maintenance of Animals. Ghost crabs (range of carapace width: 38–55 mm) were collected on South Nannygoat Beach, Sapelo Island, Georgia, from midOctober through December. Only males were used in this study. They were held at 25°C, individually, in plastic tubs containing damp sand. The sand was kept moist by daily additions of unfiltered seawater (32‰ salinity) and replaced with fresh sand when the tubs were cleaned every four to five days. The crabs were fed daily a combination of at least three of the following: Tetramin fish food, blue crab (*Callinectes sapidus*) claw muscle, mullet (*Mugil cephalus*), and live fiddler crabs (*Uca pugilator*). This diet was occasionally supplemented with bananas, chopped carrots, and lettuce.

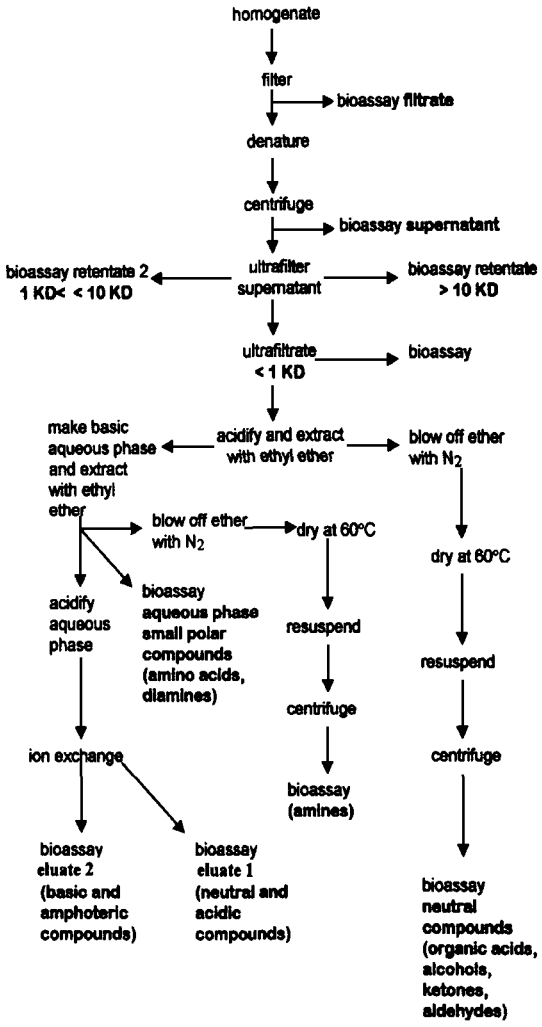

FIG. 1. Scheme depicting the procedures used to process 50-ml samples of homogenate of blue crab (*Callinectes sapidus*) claw muscle. Bioassays were conducted with 5-ml subsamples of each fraction.

The following procedures are outlined in the scheme presented in Figure 1. All glassware was acid-washed. Deionized glass-distilled water was used for solutions.

Claw Muscle Extracts. At 10°C, 50 g of muscle tissue was removed from the chelae of locally trapped blue crabs. The muscle tissue was minced in 50

ml of cold (10°C) water and diluted with artificial seawater (ASW) (Cavanaugh, 1956) to 500 ml in a 2-liter aspirator bottle. The bottle port was fitted with a self-sealing serum sleeve stopper through which samples of the homogenate were taken.

The mixture was shaken at 150 rpm for 45 min at 10°C before removing an initial 50-ml sample [time (t) = 0] with a syringe fitted with a 22-gauge needle and a 0.45-μm pore size membrane filter. The mixture was placed in a hood, kept at ambient temperature, and it was constantly stirred and aerated.

Denatured Claw Muscle Extracts. Filtered samples of the homogenate (50 ml) were taken every 12 hr for three days as the homogenate decomposed. After taking a 5-ml subsample that was frozen at −10°C, the remainder of the sample was denatured by placing it in a hot-water bath at 40°C for 45 min. Once the sample had cooled to room temperature, it was centrifuged for 15 min at 12,000g. The supernatant was frozen after recording the volume recovered. The subsamples and denatured samples were lyophilized, weighed, and stored in a desiccator under vacuum at −10°C.

Molecular Weight Fractions. The lyophilized samples were dissolved in 20 ml of water and ultrafiltered in two Amicon model 850 stirred ultrafiltration chambers at 4°C with 30 psi of N_2. They were filtered sequentially through Amicon YM-10 and YM-2 membrane filters retaining compounds with molecular weights greater than 10 and 1 kDa, respectively. Just prior to the time that a sample had passed through a filter, the membrane was washed with an additional 50 ml of 4°C water. Retentates were removed from filters by sonication.

Filtrates and retentates were lyophilized, weighed, and stored in a desiccator under vacuum at −10°C. Recombined filtrates and retentates (mix 1) served as controls and were expected to be as active as the intact homogenate in the bioassay.

Ethyl Ether Extracts. The <1-kDa ultrafiltrates were extracted by the procedure of Brooks and Moore (1960), and the various fractions were assumed to contain the classes of compounds identified in their study. Samples were dissolved in 10 ml of water, which was made acidic with 50% (v/v) HCl to pH 2. Acidified samples were first extracted with 10 ml of spectrograde ethyl ether (Fischer Chemical Company), and the ether phase containing neutral compounds (organic acids, alcohols, ketones, and aldehydes) was removed. The aqueous phase was then made basic to pH 10 with 8 N NaOH, extracted again with 10 ml of ethyl ether, and separated. The ether phase contained amines, while the aqueous phase contained small polar compounds (amino acids and diamines $<C_4$). The ether phases were blown to dryness with N_2 and the residue dried at 60°C. The aqueous phase was frozen, lyophilized, weighed, and stored with the dried ether phases in a desiccator under vacuum at −10°C. Recombined phases (mix 2) served as controls and were expected to be as attractive as the intact <1-kDa ultrafiltrates.

Ion-Exchange Eluates. The lyophilized aqueous phases were dissolved in

3 ml of water and acidified to pH 5 with 50% (v/v) HCl before applying them to a column of Dowex 50W X 8-400 (200–400 mesh, 8% cross-link; Sigma Chemical Company) ion-exchange resin in H^+ form. Nonreactive neutral and acidic compounds were washed from the resin with water (eluate 1). Basic and amphoteric compounds retained on the column were washed from the resin with 2 N NH_4^+OH (eluate 2). Both eluates were frozen, lyophilized, weighed, and stored in a desiccator under vacuum at −10°C. Combinations of the eluates (mix 3) were expected to be as stimulatory as the intact aqueous phase.

Bioassay. Test stimuli were prepared by dissolving 20 mg of lyophilized sample in 2 ml of ASW and adjusting the salinity to 32‰ with water, and the pH to 7 with either 3 N HCl or 3 N NaOH. All samples were tested at a concentration of 10 mg/ml, except neutral compounds and amines extracted in ether, which comprised approximately 1 and 1.5%, respectively, of the total weight of the extracted solutes. This small quantity of material limited the concentration tested to 2 mg/ml.

The crabs were tested by bioassay of the cheliped flexion response described in Trott and Robertson (1984). Crabs were restrained by the lateral margins of their carapace with a three-pronged flask clamp and washed thoroughly with ASW to remove sand and debris. A Kimwipe drenched in ASW was placed over the carapace and eyes to reduce visual stimulation and desiccation.

Crabs were allowed a maximum of 30 min to assume a resting position, after which their minor cheliped was stimulated with 50 μl of test solution applied with an adjustable pipet. The drop of stimulus was always applied to the dactyl–pollex joint so that it would drain down the inside edge of the dactyl. The trial was discarded if the pipet tip accidentally made contact with the claw. A positive response was scored if the cheliped was brought to the mouth where it was wiped by the maxillipeds. Positive responses were instantaneous, so trials lasted only a few seconds. Crabs were tested in groups of 10. One test lasted approximately 5 min. This was repeated three times. The mean number of responses per crab was calculated on the basis of 30 trials.

After the response to stimulation was recorded, the claw and all the appendages were washed thoroughly with ASW. Complete removal of the stimulus was assumed when cheliped flexion ceased during washing. The crabs were next tested 10 min after the previous test. Crabs were always tested with a control (ASW) following a positive response to a stimulus. Crabs never responded to ASW alone.

Statistical Treatment of Data. Significant differences in the number of responses to each stimulus fraction (factor A) [claw muscle extract, denatured claw muscle extract, ultrafiltrate (<1 kDa), retentate 1 (>10 kDa), retentate 2 (between 10 and 1 kDa), mix 1 (retentates + ultrafiltrates), polar compounds, amines, neutral compounds, mix 2 (polar compounds + amines + neutral compounds), neutral and acidic compounds, amphoteric compounds, mix 3 (neutral

and acidic compounds + amphoteric compounds)] and decomposition time (factor B) (0, 12, 24, 36, 48, 60, and 72 hr) were detected by a model I two-way analysis of variance with two repeated measures (Sokal and Rohlf, 1981). Since the two-way ANOVA detected a significant interaction between stimulus fraction and time ($F = 15.11$, $df = 66$, $P < 0.001$), significant differences between means for each stimulus at each sampling time and significant differences between means at different sampling times for a given stimulus were detected a posteriori with the Student-Newman-Keuls (SNK) multiple comparison test. This test is used to calculate q values for pairwise comparisons that are compared to "Studentized ranges" (Zar, 1996). All tests used $\alpha = 0.05$ as the level of significance.

RESULTS

Response to Muscle Extracts. Fresh muscle extract was very effective in eliciting cheliped flexion. It decreased in efficacy with progressive decomposition of the homogenate (Figure 2). After 36 hr, the stimulatory capacity of the

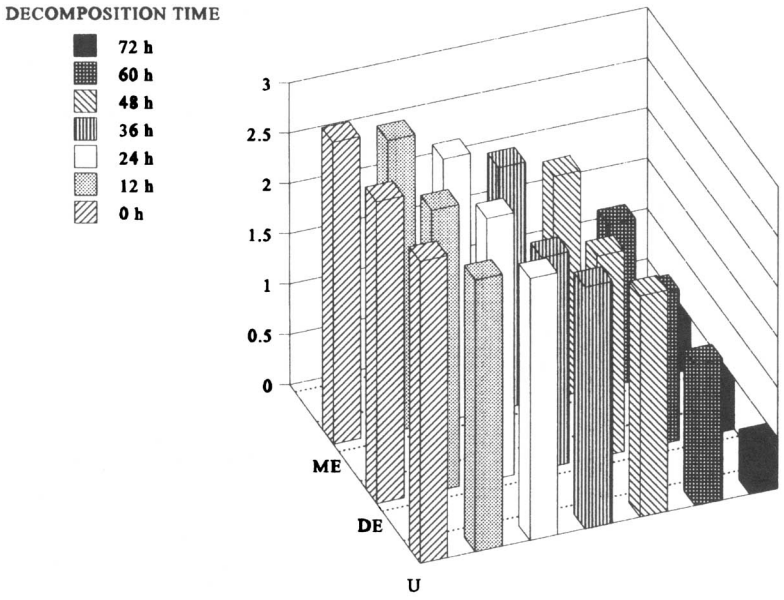

FIG. 2. Mean response of the ghost crab *Ocypode quadrata* ($N = 10$) in 30 trials to seawater extracts, intact and denatured, and ultrafiltrates (<1 kDa) of decomposing blue crab (*Callinectes sapidus*) claw muscle sampled at 12-hr intervals. ME, muscle extract; DE, denatured muscle extract; U, ultrafiltrate.

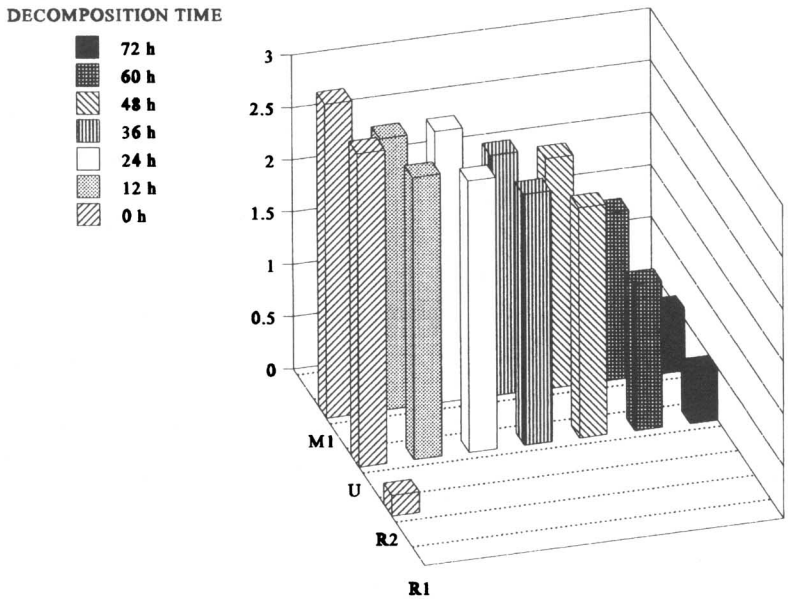

FIG. 3. Mean response of the ghost crab *Ocypode quadrata* ($N = 10$) in 30 trials to different molecular weight fractions of denatured seawater extracts of decomposing blue crab (*Callinectes sapidus*) claw muscle sampled at 12-hr intervals. R1, retentate 1 (>10 kDa); R2, retentate 2 (between 1 and 10 kDa); U, ultrafiltrate (<1 kDa); M1, mix 1 (mixture of retentates and ultrafiltrates).

extract decreased significantly ($q = 6.74$, $P < 0.05$). However, crabs continued to respond in over 50% of the trials with samples up to 60 hr old. The response to denatured muscle extract also declined after $t = 0$. The efficacy of extracts was not significantly reduced by denaturing at any stage of decomposition (Figure 2).

Response to Molecular Weight Fractions. The most behaviorally active components of muscle tissue were <1 kDa (Figure 3). The responses of crabs to compounds <1 kDa did not differ significantly from either the intact or denatured muscle extracts at any sampling time (Figure 2). Crabs did not respond to retentates >10 kDa. Retentates containing compounds between 1 and 10 kDa were ineffective stimuli also (Figure 3). When ultrafiltrates (<1 kDa) were combined with their respective low-molecular-weight fractions (mix 1), there was no significant difference in the effectiveness of this mixture from either intact or denatured muscle extracts at any sampling time.

Response to Ethyl Ether Extracts. Responses to polar compounds (amino acids and diamines $<C_4$) were significantly greater than to fractions containing

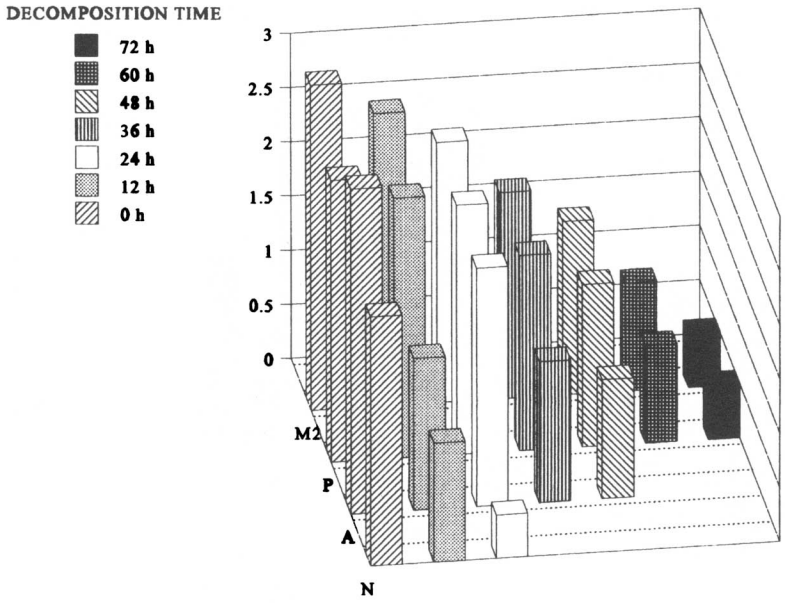

FIG. 4. Mean response of the ghost crab *Ocypode quadrata* (N = 10) in 30 trials to fractions of ethyl ether-extracted ultrafiltrates (<1 kDa) of decomposing blue crab (*Callinectes sapidus*) claw muscle sampled at 12-hr intervals. N, neutral compounds (organic acids, alcohols, ketones, and aldehydes); A, amines; P, polar compounds (amino acids and diamines); M2, mix 2 (mixture of all fractions).

neutral compounds (organic acids, alcohols, ketones, and aldehydes) at all sampling times ($P < 0.05$, Figure 4). The efficacy of polar compounds did not significantly decrease until 36 hr ($q = 4.21$, $P < 0.05$); it continued to decline throughout the remainder of decomposition (Figure 4).

The response of crabs to amines decreased significantly after 12 hr ($q = 15.38$, $P < 0.05$, Figure 4). This was followed by a significant peak in response at 24 hr ($q = 7.69$, $P < 0.05$). Crabs did not respond to amines extracted from 60- and 72-hr-old samples. Responses to fractions containing organic acids, alcohols, ketones, and aldehydes significantly decreased after 12 hr ($q = 8.57$, $P < 0.05$). Neutral compounds extracted from samples older than 24 hr did not stimulate cheliped flexion. A mixture (mix 2) of the three phases (polar compounds, amines, and neutral compounds) was as stimulatory as <1-kDa ultrafiltrates, although after 24 hr the response was significantly reduced ($q = 4.9$, $P < 0.05$).

Response to Ion-Exchange Eluates. Basic and amphoteric compounds were very effective stimulants, increasing slightly in strength but not significantly as

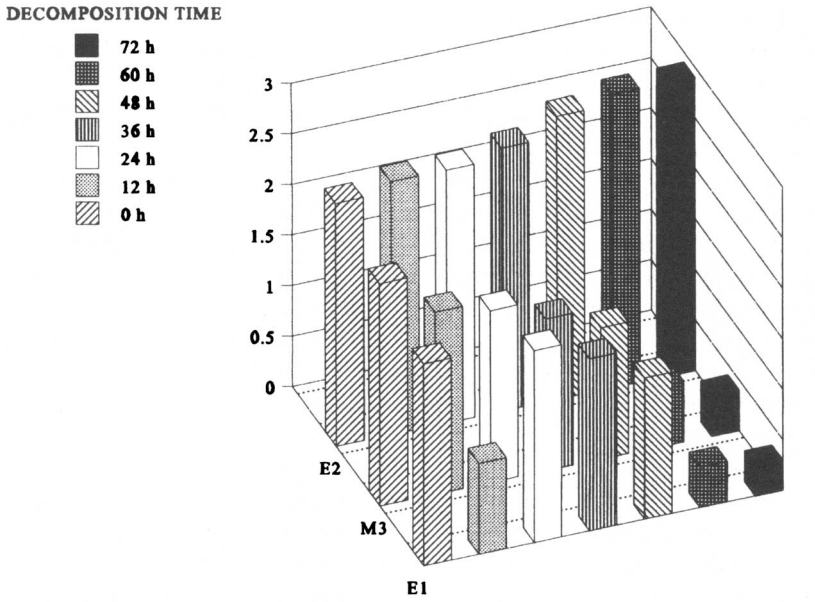

FIG. 5. Mean response of the ghost crab *Ocypode quadrata* ($N = 10$) in 30 trials to eluates from ion-exchange chromatography of aqueous phases from ethyl ether-extracted ultrafiltrates (<1 kDa) of decomposing blue crab (*Callinectes sapidus*) claw muscle sampled at 12-hr intervals. E1, neutral and acidic compounds; E2, basic and amphoteric compounds; M3, mix 3 (mixtures of both eluates).

the homogenate decomposed (Figure 5). In fact, these compounds stimulated cheliped flexion significantly more frequently than muscle extracts after 36 hr ($P < 0.05$). The responses to neutral and acidic compounds increased with time and peaked at 24 hr, when there was a significant increase in the mean number of responses per crab ($q = 6.67$, $P < 0.05$, Figure 5). The response then decreased, until by 60 hr there was no significant difference in the stimulatory capacity of neutral and acidic compounds from samples taken at $t = 0$. After $t = 12$ hr, eluates containing neutral and acidic compounds significantly ($P < 0.05$) suppressed the efficacy of basic and amphoteric compounds when combined (mix 3). The mixture was less effective than the muscle extracts after 24 hr ($P < 0.05$) and declined in efficacy with decomposition (Figure 5).

DISCUSSION

Decomposition of food made it less attractive to crabs. Fresh seawater muscle extracts were strong feeding stimulants, but with decomposition they became

significantly less attractive. This diminished efficacy of muscle extracts with tissue decay was also observed for the lobster *Homarus americanus*. The attractiveness of crude seawater extracts of codfish muscle to lobsters decreased with age (McLeese, 1973, 1974). Postlarval lobsters responded less frequently to leachates from decomposing frozen, salted, and untreated herring muscle tissue (Daniel and Bayer, 1987). The reduction in strength of seawater muscle extracts from decomposing homogenate was apparently produced by a change in the quality of the homogenate as a result of bacterial degradation. The acceptability of food may be influenced by changes in the concentrations of feeding stimulants occurring with age which would be reflected by decreased feeding responses. Feeding will not be elicited if the concentrations of feeding stimulants decrease below detection thresholds with decomposition. In addition, some crustaceans may evaluate food quality based on the ratio of ATP to ammonia; this ratio is high in fresh food and low in decomposed food (Zimmer-Faust, 1987).

The reduction in efficacy of samples taken at later stages of tissue decay may be caused by mixture suppression resulting from the production of low-molecular-weight neutral and acidic compounds. In contrast to muscle extracts, which declined in strength over time, basic and amphoteric compounds were strong stimulants throughout the course of bacterial degradation. When combined with neutral and acidic compounds from the same samples, their efficacy was significantly suppressed and over time mixtures became progressively weaker. If the nutritive value of food decreases with decomposition, mixture suppression might function to reduce the response of foraging crabs to lower quality food (Zimmer-Faust, 1987). Mixture suppression produced by combining ion-exchange eluates is unique to *Ocypode quadrata*. *Homarus americanus* does not respond to ammonia eluates (basic and amphoteric compounds) and only weakly to water eluates (neutral and acidic compounds) obtained from ion-exchange chromatography of squid extracts (Mackie and Shelton, 1972). However, when the two eluates are combined, they interact synergistically and the mixture is as effective as the whole extract.

The effect of decay was retained in the low-molecular-weight fractions. Elicitation of cheliped flexion by molecular weight fractions <1 kDa declined over time, as did the corresponding muscle extracts. McLeese (1974) also observed that the dialysate of codfish muscle extract decreased in attractiveness to *Homarus americanus* when tested at intervals up to 96 hr. This was attributed to the disappearance or transformation of material by bacteria during decomposition. Evidence from microbiological studies supports this idea. When protein is digested by bacteria, it is degraded into low-molecular-weight compounds, including amino acids, that are eventually catabolized to give ammonia and branched C_4–C_5 carboxylic acids (Elsden and Hilton, 1978). Putrefactive decomposition of beef brought about by bacteria in the genus *Clostridium* produces the diamines cadaverine and putrescine, plus significant amounts of short-

chained fatty acids, i.e., butanoic and pentanoic acids (Moore et al., 1966; Lewis et al., 1967; Brooks and Moore, 1969). These compounds are effective chemostimulants of feeding behavior in *Ocypode quadrata* (Trott and Robertson, 1984). Presumably, similar bacterial degradation products contributed to the efficacy of extracts to ghost crabs. Diamines contained in basic and amphoteric eluates may have contributed to the effectiveness of these fractions throughout tissue decay.

The most effective stimulants of cheliped flexion were low-molecular-weight compounds <1 kDa. The components of many natural or potential foods that elicit feeding behaviors in crustaceans are usually of low-molecular-weight (Carr and Guerin, 1975; Ache et al., 1976; Carr, 1978; Johnson and Ache, 1978; Rittschof, 1980; Derby, 1984). Higher-molecular-weight fractions (>1 kDa) have only limited activity in evoking feeding behaviors by crustaceans, with the exception of one species, *Panulirus interruptus* (Zimmer-Faust and Case, 1982; Zimmer-Faust et al., 1984). For *Ocypode quadrata*, retentates >1 kDa of muscle extracts were ineffective stimuli. Ghost crabs responded similarly to ultrafiltrates and retentates of mantle fluid from the ribbed mussel *Geukensia demissa* (Trott and Robertson, 1984).

Ghost crabs responded less to the two ether phases than to the aqueous phase. Two possible explanations may account for the low activity of these fractions. First, the concentrations of the test samples were five times less than that of the aqueous-phase samples. Second, on an equimolar basis, nonpolar compounds may not be very stimulatory. Mackie and Shelton (1972) found that nonpolar compounds in the lipid fraction of a chloroform–methanol extract of squid were virtually ineffective in producing feeding behavior by *Homarus gammarus*. Likewise, the lipid-containing extract of codfish muscle does not stimulate feeding responses by *Homarus americanus* (McLeese, 1974).

Ghost crabs have become successful colonizers of sandy beaches and their breadth of feeding behavior, specifically predation and scavenging, has contributed to their success in terrestrial habitats. The high efficacy of freshly injured tissues to stimulate cheliped flexion in *O. quadrata* could be an indication of a preference for freshly killed versus decomposing food. Perhaps predation is the preferred feeding behavior. In some areas of the geographical range of *O. quadrata*, this species is the top predator in a food chain composed of filter feeders, i.e., the coquina clam *Donax variabilis* and the mole crab *Emerita talpoida* (Wolcott, 1978). Ghost crabs are important scavengers on sandy beaches in addition to being predators. The diminished response of ghost crabs to decomposing muscle tissue suggests that decomposing tissues are acceptable to crabs up to a point. After 60 hr, the muscle extract still stimulated cheliped flexion in 53% of the trials. On Sapelo Island, Georgia, live prey of the type mentioned above are virtually nonexistent and scavenging is the main feeding behavior (personal observation).

Efficient chemical senses for locating and extracting high-quality food, i.e.,

nutrient and energy rich, from the environment would be selected for if an organism relies primarily on chemoreception to find food, and if its goal is to minimize the time spent foraging to maximize the net energy or nutrient intake (Charnov, 1976). Food will be rejected if normally stimulating compounds are below threshold concentrations for feeding. This study provides evidence supporting this idea. Ghost crabs responded best to low-molecular-weight compounds from fresh tissues, stimulants that may have decreased in abundance with tissue decay. Failure of decomposing tissues to stimulate feeding behavior may occur also through mixture suppression. While some components of bacterial degradation remained highly effective, their efficacy was reduced by other constituents produced during decomposition. If there is a reduction in the energy or nutrient yield of food as it decomposes, then these mechanisms for detecting compounds signaling food quality would be adaptive.

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CHARACTERIZATION OF PHEROMONE BLEND FOR GRAPEVINE MOTH, *Lobesia botrana* BY USING FLIGHT TRACK RECORDING

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Abstract—The behavioral responses of *Lobesia botrana* males to calling females, pheromone gland extracts, and synthetic sex pheromones were recorded in a wind tunnel. Gland extracts and synthetic pheromones were released from a pheromone evaporator. The numbers of males reaching the source and their flight tracks in response to calling females and pheromone gland extracts were compared to those of synthetic blends. Upwind flights to natural sex pheromone were straighter and faster than to a three-component blend of (*E*)-7,(*Z*)-9-dodecadienyl acetate (*E*7,*Z*9–12 : Ac), (*E*)-7,(*Z*)-9-dodecadien-1-ol (*E*7,*Z*9–12 : OH), and (*Z*)-9-dococenyl acetate (*Z*9–12 : Ac) (100 : 20 : 5). The optimum ratio of *E*7,*Z*9–12 : OH and *Z*9–12 : Ac to *E*7,*Z*9–12 : Ac was found to be 5% and 1%, respectively. An additional seven compounds identified in the sex pheromone gland were investigated for their biological activity. Two unsaturated acetates, i.e., (*E*)-9-dodecenyl acetate (*E*9–12 : Ac) and Δ11-dodecenyl acetate (Δ11–12 : Ac), increased the number of males reaching the source as well as straightness, linear velocity, and decreased the track angle of upwind flight. Optimum response was obtained by releasing 10 pg/min *E*7,*Z*9–12 : Ac in a mixture with 0.5 pg/min *E*7,*Z*9–12 : OH, 0.1 pg/min *Z*9–12 : Ac, 0.1 pg/min *E*9– 12 : Ac and 1 pg/min Δ11-12–Ac. The saturated acetates previously identified in the female glands were biologically inactive.

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INTRODUCTION

The main component in the sex pheromone of the grapevine moth, *Lobesia botrana* (Denis and Schiffermüller) was identified as (*E*)-7,(*Z*)-9-dodecadienyl acetate (Roelofs et al., 1973; Buser et al., 1974). Two related compounds, (*E*)-7,(*Z*)-9-dodecadien-1-ol (*E*7,*Z*9–12 : OH) and (*Z*)-9-dodecenyl acetate (*Z*9–12 : Ac), were later identified in the female sex pheromone gland and found to have a synergistic effect on the number of males landing at the pheromone source (Arn et al., 1988). Males flying to calling females steered precisely into the wind, whereas stronger deviations from the wind line were observed in response to the three-component blend (Witzgall and Arn, 1990), indicating that the three-component blend was still suboptimal. However, it was not determined whether this was due to the proportions used or that one or more essential compounds were missing from the blend.

Female moths produce species-specific sets of pheromone compounds (Linn and Roelofs, 1989). These compounds are generally shared by many species (Arn et al., 1992, 1998). Specificity of male attraction is achieved by precisely regulated proportions of blend components and by synergistic as well as antagonistic compounds (Roelofs and Wolfs, 1988; Linn et al., 1988). There is growing evidence that male flight behavior changes with pheromone blend composition (Palaniswamy et al., 1983; Willis and Baker, 1987, 1988; Quartey and Coaker, 1993; Liu and Haynes, 1993; Witzgall, 1996). Some studies have demonstrated that the closer a synthetic blend is to that produced by the calling female, the straighter and faster the males approach the odor source (Palaniswamy et al., 1983; Quartey and Coaker, 1993).

In this paper we report the results of experiments undertaken to determine if blends of synthetic pheromone components could elicit the same flight behavior from *L. botrana* males as that evoked by calling females and pheromone gland extracts. We varied the proportions of the three compounds used in previous studies and the set of sex gland compounds identified earlier (Arn et al., 1988). The main techniques used were observations in the wind tunnel and flight track recording as described by Witzgall and Arn (1990). This study was expected to be a test case to determine if flight track recording can be used as bioassay in the identification of pheromone blends.

METHODS AND MATERIALS

Insects. A culture of *L. botrana* was maintained in the laboratory on a semiartificial diet (Rauscher et al., 1984). The larvae and pupae were reared at 24°C, 18L : 6D and 65–70% relative humidity. The insects were sexed in the pupal stage. Groups of 100–150 male or female pupae were placed in cylindrical polystyrene cages (15.5 cm high × 14 cm diameter). The adult males were allowed to emerge in isolation from the females. Males and females were kept at 22 ± 2°C, 18L : 6D and 55–75% relative humidity. They were provided with water and were 2–3 days old at the time of testing.

Calling Females. Females were used during the first through the fifth hr of the scotophase. They were immobilized at 4°C, and two females were placed in a glass cartridge (18 cm long × 3.5 cm diameter). They were transferred to the wind tunnel room ca. 1 hr prior to a bioassay. The bioassay started when the females were observed calling. The light intensity at the cartridge in the wind tunnel was 20 lux.

Pheromone Extraction. Extracts of sex pheromone glands were prepared during the first hr of the scotophase. Ten glands were extracted in 100 μl ethanol at room temperature for ca. 5–10 min. Extracts were made to a stock concentration of 0.01 FE/μl. Ethanol extracts had been shown in previous experiments to be biologically more active with respect to track straightness than those made with acetone or hexane.

Chemicals. The synthetic compounds were taken from the collection of the Wädenswil laboratory. Purity was determined by gas–liquid chromatography on a DB5 capillary column (30 m × 0.32 mm, 0.25-μm film thickness, J + W Scientific, Folsom, California). The chemical purity of the tested compounds was better than 99.9% except for Δ11–12 : Ac (95%) and *E*7,*Z*9–12 : Ac (0.7% *Z*7,*E*9, 0.9% *Z*7,*Z*9, 2.6% *E*7,*E*9, overall purity 93%). The solutions were made up in HPLC grade ethanol. Vials containing the gland extracts and the synthetic compound were stored at −18°C.

Gland Extracts and Three-Component Blend. Males were tested against four release rates of the gland extracts, i.e., 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} FE/min and 0.1, 1, 10, and 100 pg/min of the three-component blend, *E*7,*Z*9–12 : Ac, *E*7,*Z*9–12 : OH, and *Z*9–12 : Ac (100 : 20 : 5). These values were chosen because the female gland of *L. botrana* contains ca. 1 ng of *E*7,*Z*9–12 : Ac as shown by Buser et al. (1974) and confirmed by our own analysis. Thus $10^{-}4$ FE/min would correspond to a release 0.1 pg/min of *E*7,*Z*9–12 : Ac.

Optimization of the Three-Component Blend. Males were flown to a two-component blend containing a constant proportion of *E*7,*Z*9–12 : Ac (10 pg/min) plus varying amounts of *E*7,*Z*9–12 : OH or *Z*9–12 : Ac, i.e., 0, 0.02, 0.1, 0.5, 2, and 10 pg/min. An additional experiment was conducted to determine the optimum ratio of *E*7,*Z*9–12 : OH and *Z*9–12 : Ac when both were combined in

a three-component blend. Males were tested against three-component blends at a constant release rate of *E*7,*Z*9–12 : Ac (10 pg/min) with varying rates of *E*7,*Z*9–12 : OH, i.e., 0, 0.1, 0.5, and 2 pg/min, and *Z*9–12 : Ac, i.e., 0, 0.1, and 0.5 pg/min.

Testing Additional Compounds. The following seven gland compounds were added to the optimized three-component blend: (*E*)–9-dodecenyl acetate (*E*9–12 : Ac), Δ11-dodecenyl acetate (Δ11–12 : Ac), decyl acetate (10 : Ac), dodecyl acetate (12 : Ac), tetradecyl acetate (14 : Ac), octadecyl acetate (18 : Ac), and eicosyl acetate (20 : Ac), 0.1, 1, 3, 0.3, 0.005, 0.0001, and 0.0001 pg/min, respectively. In the previous optimization experiments, the optimum ratio of the two minor compounds *E*7,*Z*9–12 : OH and 12 : Ac was found to be close to the ratio present in the sex pheromone gland. In line with this finding, the newly tested compounds were released at the ratio close to the amount present in the sex pheromone gland.

Pheromone Evaporator. Evaporation of the test chemicals and female gland extracts was achieved with a novel device in which a solution of the test material is dispersed as an aerosol. This is accomplished by feeding the solution through a glass capillary tube whose tip (40 μm ID) is vibrated at ultrasonic frequency by means of a piezo-ceramic disk (Cat. No. 4322 020 17621, Valvo/Philips, Hamburg, Germany). The disk is driven by a signal generator whose frequency is adjusted so that a fine mist of dispersed liquid can be observed, which, in the moving air of the wind tunnel, evaporates within a few centimeters from the tip of the capillary. The solution was delivered at a flow rate of 10 μl/min in all experiments by a microinjection syringe pump (CMA/100, Carnegie Medicine AB, Stockholm, Sweden), and the concentration was adjusted accordingly. Assuming that a droplet could be produced at each half oscillation (ca. 100 kHz) of the tip, droplets could be as small as 20 μm diameter. Our hypothesis is that with our device the dispersed solution evaporates quantitatively regardless of the vapor pressure of the individual components. Accordingly, release rates of components are given in picograms per minute. In a separate experiment, ultrasound generated during evaporation was shown to affect neither the female calling nor male flight behavior (El-Sayed, 1996).

Wind Tunnel and Recording System. We used the wind tunnel and flight track recording system of Witzgall and Arn (1991), in which the coordinates of the flying insect are recorded at video frequency (25/sec). In modification of the described system, one side of the wind tunnel was illuminated by a light box covered with three sheets of white acrylic plastic, creating a light intensity of 30 lux at the center of the tunnel, while the two cameras were placed on the opposite side at angles of 45° and 135° to the tunnel axis, respectively. The sectors covered by the two cameras overlapped over a space with a maximum length of 50 cm along the tunnel axis.

Bioassay Procedure. Males were temporally immobilized at 4°C and

groups of 25 males were transferred to glass release tubes (15 cm long × 2.5 cm diameter). The open ends of the release tubes were stoppered by rigid gauze. The tubes were taken to the wind tunnel room during the last hour of the photophase, i.e., 1 hr before the beginning of a bioassay. Bioassays commenced at the beginning of the scotophase and continued for 4 hr. Optimal response of the males was obtained during this interval (El-Sayed, unpublished). The presentation order of the treatments was reversed on alternate days. Males were released individually in the wind tunnel and the following five behavioral responses were recorded: activation (wing-fanning and walking in the release tube), take-off (beginning of flight in any direction), lock-on (beginning of upwind flight for at least 10–15 cm), close-in (upwind flight for at least 10–15 cm from the source), and landing (touchdown at the source). Timing was stopped when males landed on the tunnel walls or left the tunnel at the downwind end. Males were allowed 2 min to respond. A sufficient number of males were flown with each treatment to obtain at least 35 intact flight tracks.

Flight Track Analysis. Software especially developed for the purpose was used to analyze the flight tracks off-ilne. First, the camera coordinates were converted to 3D. Then the flight parameters were calculated as follows: linear velocity—the total distance traveled on the x axis divided by time; track angle—the deviation of the observed flight path from the wind line, calculated for every frame along each flight path; and straightness—the length of the net path traveled divided by the total distance, calculated for each flight as a whole. Incomplete flight tracks, i.e., tracks with more than three image points missing, were discarded.

Statistical Analysis. The significance of difference in the number of males completing successive stages in their advance towards the source and the flight parameters were compared by one-way analysis of variance and Duncan's new multiple-range test (StatView, 1995). Significance level was set at 0.05 for difference between the treatments.

RESULTS AND DISCUSSION

Comparing Gland Extracts to Three-Component Blend (100 : 20 : 5). At each of the four release rates tested, responses to the gland extracts were higher than to the three-component blend. The highest percentage of males landed at the source when gland extracts were released at 10^{-2} and 10^{-1} FE/min. With the three-component blend, the maximum was at 1 pg/min; further increase to 10 and 100 pg/min led to a reduction that was not significant (Figure 1). Thirty-five percent of responding males landed at the source of the three-component blend at 10 pg/min, whereas 96 and 98% landed at the source of calling females and gland extracts, respectively. Analysis of male flight tracks to call-

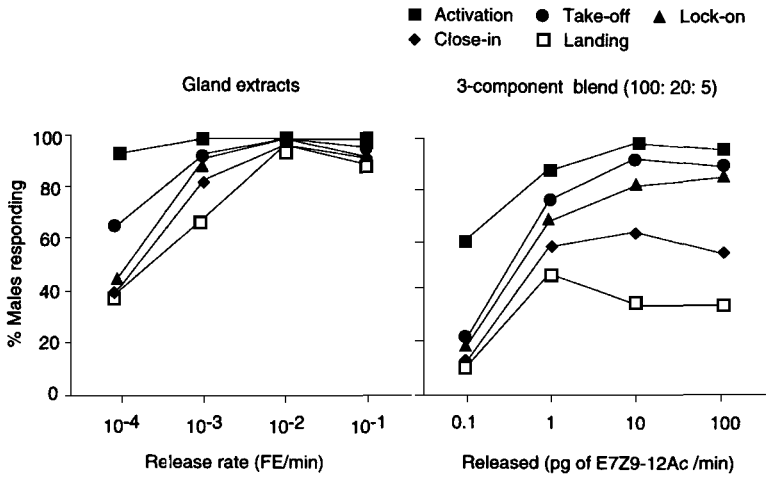

FIG. 1. Male response to four release rates of the gland extracts and 3-component blend: *E*7,*Z*9–12 : Ac, *E*7,*Z*9–12 : OH, and *Z*9–12 : Ac (100 : 20 : 5). N = 50–100.

ing females, gland extracts (10^{-2} FE/min) and the three-component blend (10 pg/min) showed distinct differences in the respective flight parameters (Table 1). Males flew significantly straighter and faster to the calling females and to the gland extracts than to the three-component blend. The track angles of males flying to calling females and the gland extracts were significantly smaller than to the three-component blend. These results show that males steered less precisely into the wind and flew at a slower speed to the three-component blend than to the gland extracts and calling females. This confirms the report by Witzgall and Arn (1990).

Optimization of the Three-Component Blend. As a first step towards optimization of the three-component blend, the optimum ratio of *E*7,*Z*9–12 : OH and *Z*9–12 : Ac to *E*7,*Z*9–12 : Ac was determined. Maximum landing was obtained when *E*7,*Z*9–12 : OH and *Z*9–12 : Ac were released at 0.5 pg/min and 0.1 pg/min, respectively (Figure 2). At higher release rates (2 and 10 pg/min) of *E*7,*Z*9–12 : OH and *Z*9–12 : Ac, males were more sensitive to *E*7,*Z*9–12 : OH than to *Z*9–12 : Ac (Figure 2). In contrast, several other tortricids, such as *Grapholita molesta* (Baker et al., 1981, Linn and Roelofs, 1983), *Adoxophyes orana* (Guerin et al., 1986), *Cacoecimorpha pronubana* (Witzgall, 1990), and *Spilonota ocellana* (Witzgall et al., 1991) showed a wide response window to the amount of synergistic alcohol components in the blend. This may be due to lower release rates of alcohols, compared to acetates, from artificial substrates such as filter paper or rubber. By comparison, the pheromone evaporator releases test solutions directly, in the formulated proportions.

TABLE 1. PERCENT LANDING AND FLIGHT PARAMETERS OF MALE *L. botrana* FLYING TO DIFFERENT SYNTHETIC BLENDS, CALLING FEMALES, AND GLAND EXTRACTS

*E*7,*Z*9–12:Ac	*E*7,*Z*9–12:OH	*Z*9–12:Ac	*E*9–12:Ac	11–12:Ac	10:Ac	12:Ac	14:Ac	18:Ac	20:Ac	Landing (%)	Flight parameters[a] Tracks (*N*)	Straightness (rel. unit)	Linear velocity (cm/sec)	Track angle (°)
10										23 a	43	0.26 ± 0.15 a	14.5 ± 05.2 a	73.1 ± 11.9 a
10	2	0.5								35 b	35	0.30 ± 0.16 a	16.6 ± 05.4 ab	69.3 ± 10.3 a
10	0.5	0.1								70 c	69	0.41 ± 0.18 b	18.7 ± 08.3 b	63.5 ± 12.0 b
10	0.5	0.1	0.1							78 cd	43	0.45 ± 0.17 bc	20.3 ± 09.4 b	61.4 ± 11.5 bc
10	0.5	0.1		1						75 cd	48	0.48 ± 0.15 cd	21.3 ± 06.7 bc	59.3 ± 10.5 c
10	0.5	0.1	0.1	1						92 e	37	0.50 ± 0.19 cd	24.1 ± 09.7 cd	57.8 ± 12.9 cd
10	0.5	0.1	0.1	1	3	0.3	0.005	0.0001	0.0001	87 de	78	0.48 ± 0.19 cd	18.8 ± 07.6 b	57.3 ± 13.3 cd
Calling females[1]										96 e	69	0.53 ± 0.16 d	26.1 ± 10.6 d	55.3 ± 11.3 de
Gland extracts[2]										98 e	90	0.57 ± 0.16 de	23.2 ± 07.2 cd	53.1 ± 10.2 e

[a]Flight parameters are mean ± Sd, values in the same column followed by the same letter are not significantly different (Duncan's new multiple range test, $P \leq 0.05$).

[b]Two calling females were used.

[c]10^{-2} FE/min, release rate of the synthetic chemicals are in pg/min. For visual observation, 75–100 males were tested for each blend.

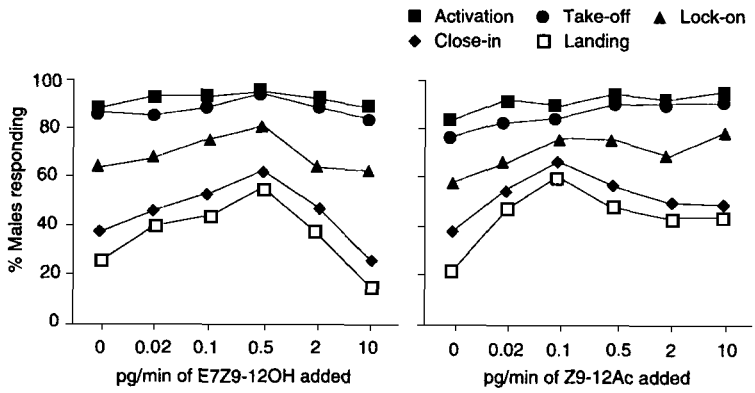

FIG. 2. Male response to two-component blends of *E*7,*Z*9–12 : Ac, released at 10 pg/min, plus varying release rates (pg/min) of *E*7,*Z*9–12 : OH or *Z*9–12 : Ac. $N = 100$.

Systematic tests with three-component blends to study the interaction between the optimum ratio of *E*7,*Z*9–12 : OH, and *Z*9–12 : Ac within the blend revealed that a blend of 10 pg/min *E*7,*Z*9–12 : Ac, 0.5 pg/min *E*7,*Z*9–12 : OH and 0.1 pg/min *Z*9–12 : Ac significantly enhanced the response of *L. botrana* males over the other ratios (Figure 3). This optimized three-component blend elicits approximately a twofold increase in activity in comparison to the previously published three-component blend (Arn et al., 1988). Analysis of the flight tracks indicated that males flew significantly straighter with smaller track angles to the optimized three-component blend than to the previously described three-component blend. There was no significant increase in linear velocity (Table 1). However, the percentage of males landing at the source and their flight parameters were inferior to those elicited by calling females or gland extracts (Table 1). The superiority of natural over synthetic sex pheromone has been reported for many species (Vetter and Baker, 1983; Sanders, 1984; Baker et al., 1991).

Testing Additional Compounds. The simultaneous presence of the two unsaturated acetates *E*9–12 : Ac and Δ11–12 : Ac with the optimized three-component blend greatly enhanced male response. Ninety-two percent of the males landed at the source when presented with this blend compared with 70% for the optimized three-component blend (Table 1). On the other hand, there was no significant difference between the three-component blend and the two four-component blends. Male response to the five-component blend was equivalent to the 10-component blend containing the saturated acetates. The flight tracks of males responding to the five-component blend were significantly faster than to the optimized three-component blend. Males also flew with significantly smaller track angles to the five-component blends than to the optimized three-compo-

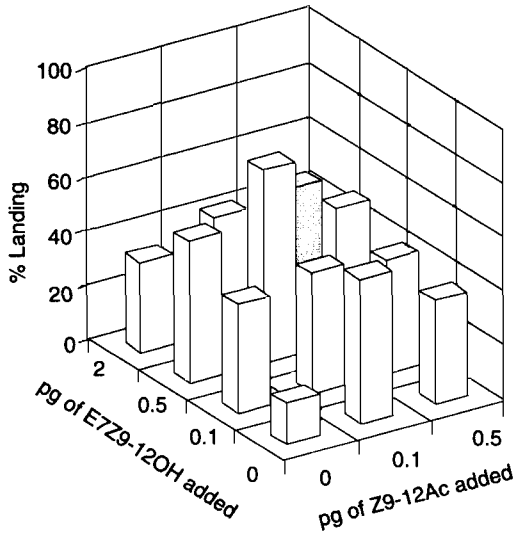

FIG. 3. Males landing at three-component blends of *E*7,*Z*9–12 : Ac, released at 10 pg/min plus varying release rates (pg/min) of *E*7,*Z*9–12 : OH and *Z*9–12 : Ac. The shaded column corresponds to the previously established three-component blend (100 : 20 : 5) of Arn et al. (1988). N = 75–100.

nent blend. The presence of 10 : Ac, 12 : Ac, 14 : Ac, 18 : Ac, and 20 : Ac, with the five-component blend did not improve any of the flight parameters, and the linear velocity was significantly decreased. The biological activity of the five-component blend, evaluated by the number of males landing at the source and their flight parameters, was similar to the gland extracts except for the track angle, in which the difference was significant (Table 1). This suggests that the minor pheromone compounds, *E*7,*Z*9–12 : OH, *Z*9–12 : Ac, *E*9–12 : Ac, and Δ11–12 : Ac are part of the biologically active sex pheromone components of *L. botrana*.

Flight Track Analysis. The validity of track analysis compared to the conventional recording of behavior is shown in the plot of males landing at the source versus the three flight parameters, giving good correlation (Figure 4). The Spearman rank-correlation coefficient, r_S = 0.97 for the straightness, r_S = 0.88 for the linear velocity and r_S = 0.97 for the track angle indicate a linear relationship.

Track recording has proven to be a more sensitive tool to identify the role of additional components involved in the mating signal than simple counting of the number of males landing at the source. For example, there was no significant difference between the number of males arriving at the source among the best

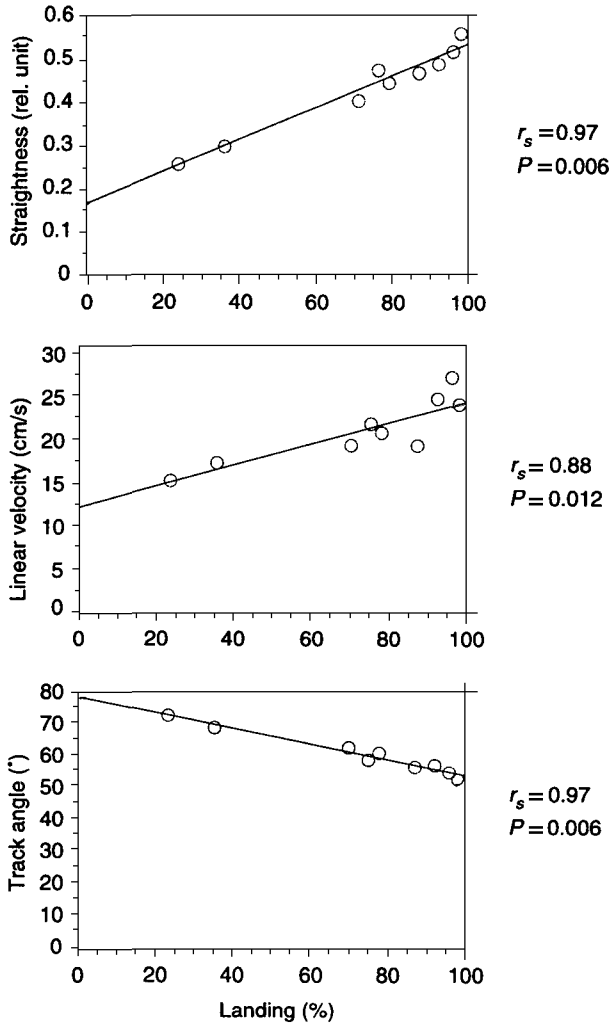

FIG. 4. Correlation between males landing at the source and the three flight track parameters used for assessing blend quality. Spearman's rank-correlation coefficient and the corresponding significance is given for each relationship.

four lures in Table 1. However, analysis of the flight tracks indicated that males flew with significantly lower linear velocity to the 10-component blend than to the other three lures. This showed that the presence of the five saturated acetates did not affect the number of males landed at the source but affected the flight

speed. Track analysis data showed that males flew with significantly smaller track angle to the gland extracts than to the five-component blends. This might indicate that the synthetic blend is still incomplete.

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ECOLOGICAL EXAMPLE OF CONDITIONED FLAVOR AVERSION IN PLANT–HERBIVORE INTERACTIONS: EFFECT OF TERPENES OF *Eucalyptus* LEAVES ON FEEDING BY COMMON RINGTAIL AND BRUSHTAIL POSSUMS

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Abstract—We investigated the hypothesis that feeding deterrence of common ringtail possums (*Pseudocheirus peregrinus*) and common brushtail possums (*Trichosurus vulpecula*) by *Eucalyptus* terpenes (in this case 1,8-cineole) is a result of a conditioned flavor aversion (CFA), due to the association of terpenes with postingestive effects of another group of *Eucalyptus* toxins, the diformylphloroglucinol compounds (DFPCs). Wild-caught common ringtail and common brushtail possums showed a dose-dependent reduction in food intake when 1,8-cineole was added to the diet. However, after continued exposure over 12 days to increasing amounts of cineole in the diet, both species substantially increased their intakes of cineole relative to control animals. This indicated that the aversion to cineole was a conditioned response rather than a physiological limitation in their ability to detoxify terpenes. Subsequent exposure to a diet including both cineole and jensenone (a simple DFPC also found in *Eucalyptus* and considered to cause postingestive emesis) in corresponding amounts was able to recondition the dose-dependent aversion. Consequently, animals that had been given jensenone showed an aversion to cineole-rich diets that matched the behavior of animals in the control group. This supported our hypothesis that the effect of terpenes on feeding of these marsupial folivores on *Eucalyptus* is due to a CFA. Possums can cope with levels of terpenes in the diet that far exceed those occurring naturally. The role of terpenes in marsupial folivore–*Eucalyptus* interactions

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appears to be to act as a cue to levels of toxic DFPCs in the leaves, rather than acting as toxins in their own right.

Key Words—Conditioned flavor aversion, plant toxin, *Eucalyptus*, *Pseudocheirus perigrinus*, *Trichosurus vulpecula*, terpene, cineole, jensenone, food intake, emesis.

INTRODUCTION

Conditioned flavor aversions (CFAs) are considered to be an important mechanism modulating diet choice in free-ranging herbivores (Provenza, 1996). Animals are thought to regulate their intake of some plant secondary metabolites (PSMs) below toxic levels by learning to associate a distinctive taste of a diet with negative postingestive feedback resulting from the PSM. Studies of this phenomenon in browsing mammals mostly have been conducted with ruminants and have used toxins (principally LiCl) (Wang and Provenza, 1997) and/or artificial flavors such as orange or onion as the taste stimulus (Ralphs et al., 1995; Wang and Provenza, 1997), which do not occur naturally in the diets studied. Although these studies have shown how CFAs could develop in free-ranging animals, the stimuli used are not ecologically realistic. In contrast, Kyriazakis et al. (1997) used two naturally occurring plant secondary metabolites (PSMs) to condition aversions in sheep, but again they used flavor stimuli that the animals are unlikely to encounter in nature. In this study we examine the development of a conditioned flavor aversion in the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*), both arboreal marsupial folivores of *Eucalyptus*. We used both a flavor stimulus (1,8-cineole, a monoterpene) and a toxin (jensenone, a diformylphloroglucinol compound; see below) which occur together in *Eucalyptus* leaves.

It has been suggested that terpenes deter feeding of a wide range of browsing mammals, including the marsupial folivores of *Eucalyptus* (Southwell, 1978; Hume and Esson, 1993) because small amounts can be toxic to some animals (McLean and Foley, 1997). However, correlations between food intake and the concentrations of foliar terpenes have explained little, if any, of the differences in foliage intake by these animals (Southwell, 1978; Hume and Esson, 1993). In contrast, we showed that intraspecific variation in food intake by common ringtail possums correlates strongly with the total terpene concentration of the leaves (Lawler et al., 1998a). However, in these experiments, food intake also correlated with the concentration of a newly discovered group of compounds, the diformylphloroglucinol compounds (DFPCs). A strong correlation between food intake and foliar DFPC content has now also been found with two other species of *Eucalyptus* (Lawler, Foley, Eschler, unpublished data).

We suggested that foliar terpenes could not cause the variation in intakes

at the concentrations observed between individual trees (Lawler et al., 1998a). When added to an artificial diet, the terpene concentration required to reduce food intake was far greater than that found in leaves on which food intakes were reduced to similar levels. In contrast, addition of DFPCs to the diet reduced intakes at levels in close agreement with the trend seen in leaves. This finding was supported also by more recent studies of two other *Eucalyptus* species (Lawler, Foley, Eschler, Schliebs, unpublished data). Consequently, we suggested that foliar terpenes may cue animals to the concentration of the true toxins in the leaves. Terpenes have strong and distinctive odors and tastes and are responsible for the characteristic smell of *Eucalyptus* leaves, while DFPCs are nonvolatile and give off very little noticeable odor (Lawler, personal observation). All DFPCs contain a side chain derived from common terpenes (such as β-phellandrene and bicyclogermacrene) (Ghisalberti, 1996), and so we suspected that concentrations of the two compounds would be correlated if production of DFPCs is substrate-limited (Lawler et al., 1998a). Such correlations have now been found in three *Eucalyptus* species (Lawler and Foley, unpublished). Upon initial inspection and tasting of a leaf, a folivore would first encounter information about the terpene components of the leaf and might use this to estimate the DFPC content of the leaves and its likely postingestive effects, based on previous experience. Indeed, we have found that even with our relatively limited olfactory capacity, we could predict the relative amounts of DFPCs between foliage samples of different individual trees within *Eucalyptus* species, simply by crushing and smelling the leaves upon collection (Lawler and Foley, unpublished).

In this study we aimed to test the following three hypotheses: (1) Wild-caught folivorous possums held in captivity will show a strong aversion to *Eucalyptus* terpenes, due to their previous experience. (2) This aversion can be reduced or removed by feeding the animals an artificial diet to which increasing amounts of terpene are added. (3) Once removed, the aversion can be reconditioned by giving the animals a diet consisting of both the terpene and another *Eucalyptus* toxin known to produce negative postingestive effects in corresponding concentrations.

METHODS AND MATERIALS

This research was approved by the Animal Experimentation Ethics Committee of the Australian National University and conforms with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twelve common ringtail (*Pseudocheirus peregrinus*) and 12 common brushtail (*Trichosurus vulpecula*) possums were collected from *Eucalyptus* woodlands, kept individually in metabolism cages (described by Lawler et al., 1998a), and maintained on a basal diet made fresh each day. The diet fed to ringtail possums consisted of (percent

wet matter) 55.5% grated apple, 28.3% banana pulp, 4.7% ground lucerne hay (to pass a 2-mm sieve), 5.5% ground rice hulls, 4.7% ground Weetbix (a wheat-based breakfast cereal), and 1.6% acid casein. The diet for common brushtails was modified for the purposes of other experiments and consisted of 55% apple, 15% carrot, 15% banana, 5.8% sugar, 1% lucerne hay, 3% Weetbix, 5% rice hulls, and 0.2% casein. All animals maintained body mass on this basal diet.

For each experiment, a *Eucalyptus* terpene (1,8-cineole) and a simple DFPC (jensenone) (Boland et al., 1992) were added to the diet in varying concentrations and aversion/deterrence measured as a reduction in food intake relative to control values. No alternative food was offered during experimental periods. For ringtail possums, all experimental periods were separated by a day on which only the basal diet was offered, in order to reduce carryover effects and ensure the welfare of the animals. This was not considered necessary for brushtail possums as they readily feel during the day and so were offered an untreated diet between 08:00 hr and 12:00 hr on each experimental day, which similarly reduced carry over effects and ensured the health of the animals.

Cineole was chosen as a representative terpene as it is one of the most common terpenes found in *Eucalyptus*, often dominating the total volatile terpene extract (Boland et al., 1991), and has specifically been suggested as a feeding deterrent in studies of both mammals (Southwell, 1978; Reichardt et al., 1990; Zoidis and Markowitz, 1992) and insects (Edwards et al., 1993). Cineole was purchased from Sigma Chemical Company, Australia.

Jensenone was used as a representative DFPC as it is structurally similar to other active DFPCs, is active in similar molar quantities (Lawler et al., 1998a), and is the only DFPC that we have thus far been able to isolate in sufficient quantity for these kinds of experiments (Lawler et al., 1998b). We also have evidence that jensenone acts by stimulation of the emetic system, as much of its effect on intake can be attenuated by administration of ondansetron (Lawler et al., 1998b), an antiemetic drug that is a $5HT_3$ receptor antagonist (Butler et al., 1988). In this way it is similar to LiCl, the emetic stimulant so frequently used in other studies (Launchbaugh and Provenza, 1993; Provenza et al., 1994b; Wang and Provenza, 1997). Jensenone was extracted from air-dried foliage of *Eucalyptus jensenii*, ground to pass a 2-mm sieve, and extracted in 20% acetone–light petroleum in a Soxhlet apparatus. The extracts were concentrated and combined in diethyl ether and washed with 0.3 M NaOH. These washes were then acidified with HCl, and the precipitate was washed with ethanol and recrystallized from acetone to give jensenone (98% by ^{1}H NMR) (Eschler and Foley, unpublished data).

As cineole is volatile, it was added to the diet by adsorbing it onto the dry components immediately before adding the fruit. Jensenone is nonvolatile and insoluble in water, so it was dissolved in acetone and then added to the dry component of the diet, after which the acetone was evaporated before mixing with the fruit component of the diet. Control diets were treated similarly with acetone.

Independence of Effects of Cineole and Jensenone in Common Ringtail Possums

As this experiment relied on our ability to predict the amount of reduction in food intake associated with jensenone and cineole together, it was necessary to investigate firstly whether there was any interaction between the effects of the two compounds before attempting to remove any aversion to cineole. In this experiment, carried out on ringtail possums, there was no significant interaction, so we considered it unnecessary to also conduct this experiment with brushtail possums as their reactions to both compounds are similar (Lawler et al., 1998b; Stapley, unpublished).

Three concentrations of each compound were used. Cineole was added to the diet at concentrations of 0, 6.3, and 9.8% of total dry matter (DM) and jensenone was added at 0, 0.84, and 1.26% of the total DM of the diet. In an orthogonal design this resulted in nine possible treatment combinations. A truncated Latin-square design was used with nine possums over five treatment periods, so that over the experiment each possum received five different randomly allocated treatments.

Removal and Reconditioning of Aversion to Cineole

The experiments with the ringtail possums and brushtail possums were carried out separately, with those on ringtail possums conducted earlier. Following the ringtail possum experiments, some adjustments to experimental design were made for the brushtail possums and differences in the protocols (principally for stages 1 and 3) are listed below. For both species the animals were divided randomly into two groups of six (a control and a treatment group) and the experiments were carried out in five sequential stages. The complete series of experiments took 35 days for ringtail possums and 27 days for brushtail possums.

For clarity, the five stages of the basic design and the expectations and hypotheses are summarized here, with full details of each experiment given below. In stage 1—initial/preacclimation aversion to cineole—the ability of both groups to ingest cineole in no-choice experiments was tested. The hypothesis to be tested is that both groups should show a similar strong reduction in food intake when cineole is added to the diet. In stage 2—acclimation to cineole—the treatment group was acclimated to increasing levels of dietary cineole over 12 days while the control group was fed only the basal diet. The expectation is that animals in the treatment group will increase their intake to cineole to levels well beyond the tolerance shown in stage 1. In stage 3—postacclimation to cineole—the tolerance of each group to cineole was again tested, and animals in the treatment group were expected to show significantly less reduction in food intake than the animals in the control group in response to dietary cineole. In stage 4—reconditioning aversion to cineole—the aversion was reconditioned by giving

the treatment group a diet including both jensenone and cineole in corresponding concentrations. The hypothesis is that jensenone should reduce food intake to levels similar to those expected on the basis of the initial aversion to cineole. In stage 5—postreconditioning aversion to cineole—the tolerance of both groups to added cineole (without jensenone) was again tested, and we expected that the reduction in food intake due to dietary cineole (in the absence of jensenone) should be similar to that seen in the control group and be dose-dependent.

Stages 1 and 3. In ringtail possums tests of the ability of the two groups of animals to tolerate cineole were carried out as crossover designs involving two treatments and two periods. On the first day, three animals in each group were given diets of 9.8% cineole on a dry matter (DM) basis, while the remaining three were given the basal diet alone. The following day all animals were fed the basal diet and the next day the treatments were reversed.

In brushtail possums similar tests were made with three concentrations of cineole (0, 4.9, and 9.8% DM). Animals in both the treatment and control groups were divided into two subgroups and the diets offered in a 3×3 Latin-square design to each group of three animals over three consecutive nights. A more extensive design with more concentrations was avoided as it was thought that there may be some loss of the aversion in a longer experiment.

Stage 2. Six animals were acclimated to increasing levels of cineole in their diets while the remaining six were fed the basal diet only. The initial concentration of cineole was 1.75% DM; this was increased by approximately 0.7% DM per day to a maximum of 10.5% DM for ringtail possums and by approximately 1.2% DM per day to a maximum of 16.5% DM for brushtail possums. Stages 2 and 1 were necessary as the animals were caught from *Eucalyptus* woodland and their intakes of foliage diets had been shown to correlate strongly with leaf terpene concentrations (Lawler, unpublished); hence, they were likely to already have a CFA towards cineole. That is, it is possible that the animals had already made associations between cineole or some other foliar terpene and diet choice. This possibility had to be evaluated and its influence removed in the later stages of the experiment. It was not possible to find possums that we could confidently say had not been exposed to terpenes in their natural diets.

Stage 4. The treatment group was given the basal diet to which cineole and jensenone was added in corresponding concentrations, that is, concentrations of each compound, which, on the basis of the first experiment described above (for ringtails or Stapley's unpublished data (for brushtails) would produce the same level of reduction in food intake. The amount of jensenone added to the cineole-rich diet was that required to reduce food intake to the same level as seen when cineole alone was incorporated before removal of the aversion. The purpose of this was to allow the animals to learn to associate the concentration of cineole with a given level of postingestive discomfort. Three concentrations of each compound were used. These were (jensenone/cineole as %

DM): 0.42/4.39, 0.84/8.78, and 1.26/13.17 for ringtail possums and 0.176/4.5, 0.29/7.5, and 0.43/9.8 for brushtail possums.

A 6 × 6 Latin-square design was used to allocate treatments among the six animals, so that each animal was given each concentration twice over the course of the experiment.

Stage 5. For both species, three treatment levels were used as described above for brushtail possums in stages 1 and 3. This procedure of using stages 1 and 3 for ringtails (and all stages for brushtails) was done in order to show that any reconditioned aversion was also dose-dependent, rather than a simple avoidance. It was not initially considered necessary to examine dose-dependence for the previous cineole aversion of ringtail possums as this was shown in the experiment that tested for interactive effects of jensenone and cineole.

Statistical Analysis

All experiments were treated as mixed model, row–column designs with possums and days as random effects and cineole concentration, group (treatment or control), and group × cineole interaction as fixed effects. Variables of the models were estimated by restricted maximum likelihood (REML) theory (R. Cunningham, personal communication). For the key experiments, data presented are for the effect of cineole on DM intake and the cineole × group interaction effect on DM intake. We expected that cineole would reduce DM intake and, where the aversion to cineole was similar between control and treatment groups (stages 1 and 5), the interaction would be nonsignificant, while where the treatment group had a reduced aversion, relative to the controls, the interaction would be highly significant (stage 3).

Data Presentation

All data are presented graphically. Where formal statistical tests have been performed (stages 1, 3, and 5) means are presented with a bar showing least significant difference (LSD) derived from the analysis. Where we have not performed formal tests of differences (stages 2 and 4), data are shown as means ± standard errors.

RESULTS

Independence of Effects of Cineole and Jensenone in Common Ringtail Possums

There was a significant interaction between dietary cineole and jensenone on food intake of common ringtail possums (ANOVA: $P < 0.001$). However, this was mainly attributable to the overriding influence of jensenone on food intake,

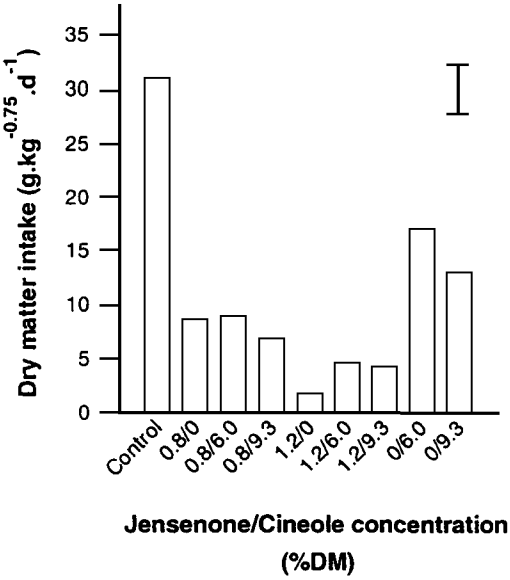

FIG 1. Effects of combinations of 1,8-cineole and jensenone on food intake of common ringtail possums. Data are means with least significant difference in the analysis represented by the bar in the upper right part of the figure. Food consumption is based on dry matter (DM) intake.

rather than any strong additive or synergistic effect of the two compounds combined (Figure 1). Thus, the combination of the two in the reconditioning of any CFA was considered appropriate.

Removal and Reconditioning an Aversion to Cineole

The salient data are given in Figure 2, with data for ringtail possums listed on the left (Figure 2a) and those for brushtail possums on the right (Figure 2b). Statistical tests are summarised in Table 1. In summary, both species showed an

FIG. 2. Effects of 1,8-cineole on food intake of (a) common ringtail possums and (b) common brushtail possums at different stages in the experimental sequence. [i] Initial/preacclimation aversion after capture from *Eucalyptus* woodland; [ii] postacclimation of animals in the treatment group to increasing dietary cineole; [iii] postreconditioning of the aversion in animals in the treatment group by combining added dietary cineole and jensenone in corresponding amounts. Shaded bars represent the control group; unshaded bars represent the treatment group. Data are means with least significant difference in the analysis represented by the bar in the upper right part of the figure.

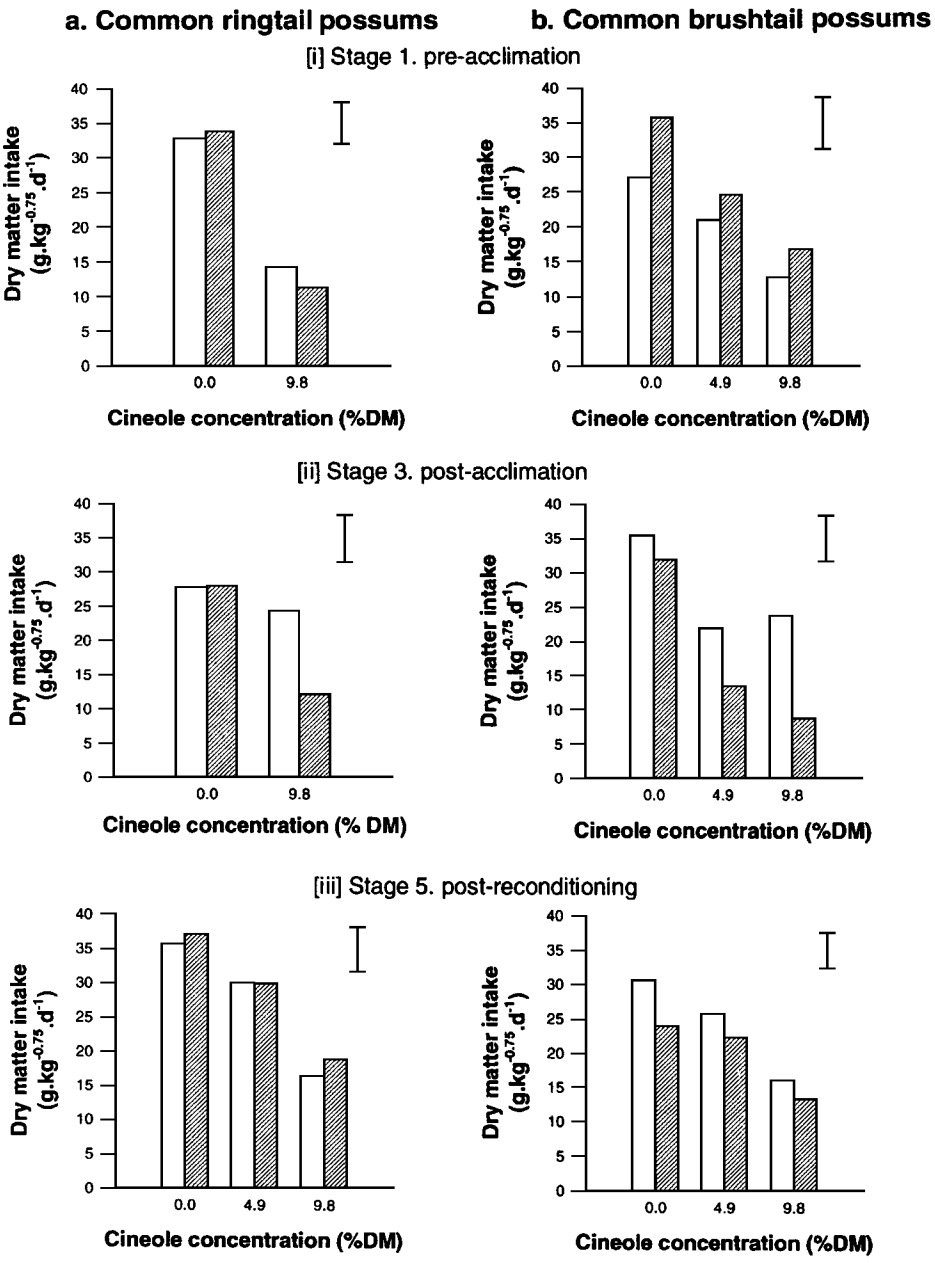
a. Common ringtail possums
b. Common brushtail possums
[i] Stage 1. pre-acclimation
[ii] Stage 3. post-acclimation
[iii] Stage 5. post-reconditioning
Dry matter intake (g.kg-0.75.d-1)
Cineole concentration (%DM)
Cineole concentration (% DM)
0.0
4.9
9.8

TABLE 1. SUMMARY OF PROBABILITY VALUES FROM STATISTICAL ANALYSES OF COMPARISON OF AVERSION TO 1,8-CINEOLE BETWEEN ANIMALS IN CONTROL AND TREATMENT GROUPS

	Ringtail possums		Brushtail possums	
Experiment stage	Cineole main effect	Cineole × treatment interaction	Cineole main effect	Cineole × treatment interaction
Preacclimation	<0.001	0.403	<0.001	0.472
Postacclimation	<0.001	0.001	<0.001	0.02
Postreconditioning	<0.001	0.778	<0.001	0.861

initial aversion to cineole (stage 1: Figure 2[i]), which was substantially reduced after continued exposure (acclimation) to cineole (stage 3: Figure 2[ii]) and was reconditioned after exposure to dietary cineole with corresponding amounts of dietary jensenone (stage 5: Figure 2[iii]). Results for each stage [including intermediate acclimation (stage 2) and reconditioning (stage 4)] are described fully below.

Stage 1. Initial/Preacclimation Aversion to Cineole. Common ringtail possums in both treatment and control groups showed a clear initial aversion to dietary cineole, which was consistent between groups (Table 1, Figure 2a[i]). Common brushtail possums showed a similar response to both cineole as a main effect and the cineole by group interaction (Table 1, Figure 2b[i]).

Stage 2. Acclimation to Cineole. Both common ringtail and common brushtail possums substantially increased their intakes of cineole over the acclimation period (Figure 3a and b).

Stage 3. Postacclimation Aversion to Cineole. After acclimation to dietary cineole, both species showed substantially reduced aversions relative to animals that were not acclimated. There was still a significant main effect of cineole, due principally to the remaining strong aversion in the control group (Table 1, Figure 2a and b[ii]).

Stage 4. Reconditioning of Aversion to Cineole. During the reconditioning phase of the experiment, animals of both species clearly regulated food intakes in relation to the jensenone (and hence cineole) concentration of the diet (Figure 4a and b).

Stage 5. Postreconditioning Aversion to Cineole. After reconditioning, the aversions of animals to cineole in the treatment groups for both species matched those of the animals in the control groups that had never had an opportunity to learn that cineole is not always associated with negative postingestive feedbacks (Figure 2c[i] and [ii]).

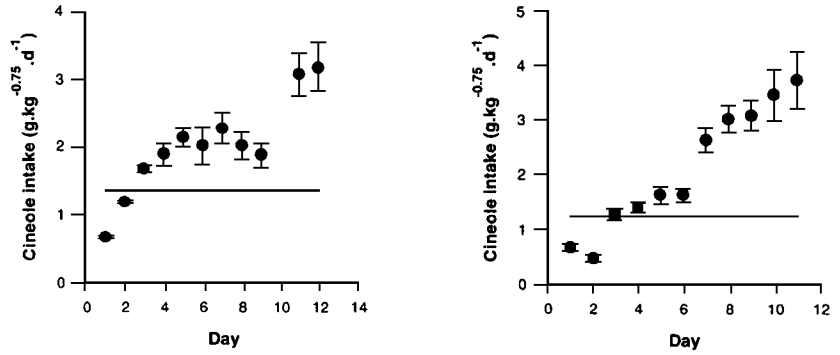

FIG. 3. Intake of 1,8-cineole by common ringtail possums (left) and common brushtail possums (right) during acclimation to increasing dietary cineole. Solid lines represent previous mean maximum intake of cineole by the same animals. Data are means ± standard errors.

DISCUSSION

The data presented here show that the feeding deterrence of terpenes in *Eucalyptus* is mediated largely through a conditioned flavor aversion. This aversion can be removed and then reconditioned by dissociating the taste and toxic stimuli and then reinstating the association. Several studies have examined the

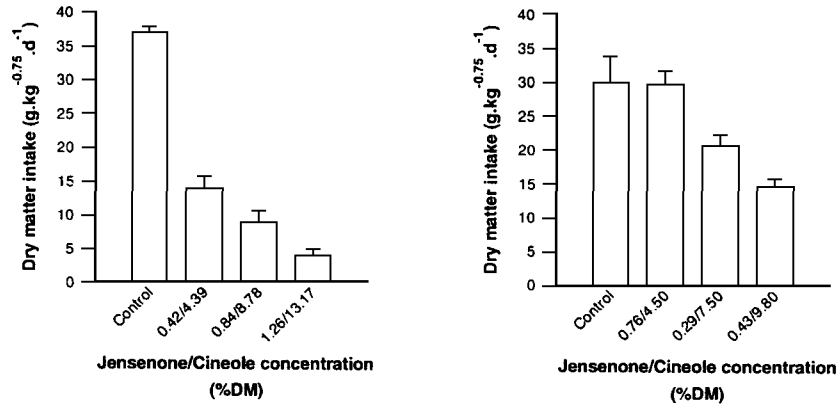

FIG. 4. Effect of 1,8-cineole and jensenone in combination on food intake of treatment animals only for common ringtail possums (left) and common brushtail possums (right). First column shows intakes of animals in the control group concurrent with measured intakes of animals in the treatment group. Data are means ± standard errors.

conditions required to produce a flavor aversion in mammals, but we believe that this is the first study to identify both a salient taste and a toxin that influence a natural plant–herbivore interaction. In studies of browsing ruminants, there have been examples of identified aversive compounds (Kronberg et al., 1995; Provenza et al., 1994a), and it has been suggested that the degree of aversion may relate to the concentration of a salient flavor; however, the compound responsible for the flavor was not identified (Provenza et al., 1994a). It has also been shown that, when flavor and toxins are highly correlated, herbivores learn to regulate food intake based on interactions between postingestive feedback and flavor and then adjust intake on the basis of changes in flavor alone (Launchbaugh et al., 1993).

In *Eucalyptus*, the underlying negative feedback is caused by the DFPCs and this causes an aversion to the taste of terpenes, which occur in concentrations correlating with the DFPCs in the three eucalypt species studied to date (Lawler, Foley, Eschler, unpublished data). The aversion to the taste of cineole is dose-dependent: animals eat less of diets with high concentrations of cineole, apparently conditioned to a potentially greater "internal malaise" (sensu Provenza, 1995). It has been shown that one cause of CFAs is stimulation of the emetic system of the body (Provenza et al., 1994b). This appears to be at least part of the mechanism for conditioning an aversion due to jensenone (and, we suspect, other DFPCs). Administration of an antiemetic drug (ondansetron, an antagonist of the $5HT_3$ serotonin receptors) allowed both common ringtail and common brushtail possums to significantly increase their intake of a jensenone-treated diet relative to animals given only the treated diet (Lawler et al., 1998b).

The role of terpenes in deterrence of mammalian folivores of *Eucalyptus* appears to have been overstated (Lawler and Foley, 1998). They appear not to be the primary cause of the deterrence, but rather their strong, overriding smell and taste serve as a cue to levels of the true deterrent compounds. Whereas it has been shown that there are physiolgoical costs associated with the detoxification of terpenes by these animals (Foley, 1987, 1992), no study to date has examined whether these costs are indeed limiting to food intake of the animals (Foley and MacArthur, 1994). The limiting cost would probably not be reached in the natural diet, in which concentrations of terpenes rarely exceed 5% (Hume and Esson, 1993; Lawler et al., 1998a), since this was clearly not deterrent to either the common ringtail possums or the brushtail possums in this study. In contrast, previous work (Lawler et al., 1998a) and ongoing work (Lawler, unpublished) show that the DFPCs can deter marsupials from eating when applied to food in ecologically realistic concentrations.

Assays of the terpene content of leaves may still be useful if we use them as do the animals, as a de-facto indicator of leaf toxicity. The DFPCs have been detected in 38 of 40 *Eucalyptus* species examined (Eschler, Pass, Foley, unpublished). In the three eucalypt species studied in detail to date, the concentrations

of the two groups of compounds are closely correlated (Lawler, Foley, Eschler, unpublished data). However, the threshold DFPC (and terpene) concentration that results in a reduction in food intake may vary between different species of *Eucalyptus*. Whereas the terpene and DFPC concentration of the foliage within a single species correlate strongly, the slope of this relationship appears to vary significantly between species (Lawler, unpublished data). Furthermore structural diversity of DFPCs is such that the biological activities of the different DFPCs may vary as well. The biosynthesis of the DFPCs and the biochemical relationships between the DFPCs and terpenes is an area needing further study.

This begs the question of whether high concentrations of terpenes can confer resistance to herbivores in the absence of DFPCs. Can susceptible plants of the same or different species as resistant ones use mimicry to escape herbivory? Launchbaugh and Provenza (1993) concluded that flavor mimicry could reduce the amount of damage that herbivores inflict on plants if those plants had a similar taste and odor to genuinely resistant plants. It is conceivable that plants rich in terpenes but poor in DFPCs could gain some measure of protection from marsupial folivores, especially if the different types (low or high DFPC) are individuals of the same species, with very similar terpene profiles. However, CFAs are rapidly extinguished under a range of conditions, and there are restrictions also on the effectivenes of mimicry, which are considered below.

While the long-lived nature of CFAs has been emphasized in the past, recent work emphasizes the dynamic nature of foraging in environments where toxins and nutrients are spatially heterogeneous, requiring constant reassessment of the consequences of ingestion of particular foods (Wang and Provenza, 1997). Repeated exposure to the flavor stimulus alone leads to rapid reduction in the strength of the aversion (O'Connor and Matthews, 1997; this study), and several situations may arise that encourage animals to resample a diet possessing a flavor that previously led to an aversion. Where the amount of nonaversive food is limited, hunger will force the animals to sample aversive foods (Provenza, 1996), and, in fact, common ringtail possums continued to sample a diet containing toxins even when a toxin-free control was available *ad libitum* (D. Pass, personal communication). In free-ranging animals the social context is also important. For example, when associated with animals not possessing the aversion, averted cattle lose an aversion more rapidly (Ralphs, 1997).

Mimicry frequently requires a high proportion of the toxic model before the mimic gains significant advantage (e.g., Lindstrom et al., 1997). It seems likely that flavor mimicry in plants would be an effective strategy to avoid herbivory only when the proportion of mimics in the habitat is very low relative to truly toxic plants of the same flavor. This would be especially true where the herbivore can also incorporate spatial information about the models and the mimics. It is likely also to be necessary to have an abundance of alternative palatable food available to prevent the animals from needing to sample unpalatable items. Ter-

penes themselves are therefore unlikely to constitute an effective defense against folivorous possums.

The concepts discussed above may prove useful in the management of folivorous possums where they occur as pests of commercial *Eucalyptus* plantations. Commercial operations have suffered significant losses due to predation of trees by marsupials (Montague, 1994), as well as from chewing insects. The use of genotypes that are naturally resistant to herbivores may be one means of managing these losses, and entomologists have suggested selecting for cineole-rich genotypes that may deter some insect herbivores (Edwards et al., 1993; Farrow, 1993). This may be successful in providing cross-resistance against marsupial browsers in the short-term but, given our current lack of knowledge of the biosynthesis of the DFPCs (Ghisalberti, 1996), we cannot safely assume that selection for high terpene plants will always result in high DFPC plants. Considerable effort may be expended on producing genotypes that have substantial leaf terpenes but insufficient DFPCs to deter marsupial folivores in the longer term. Folivorous marsupials living within or adjacent to these kinds of trees may quickly lose any aversion based on the taste of terpenes.

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URINARY VOLATILE PROFILES OF THE DEERMOUSE (*Peromyscus maniculatus*) PERTAINING TO GENDER AND AGE

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Abstract—Biological aspects of chemical communication in the deermouse have been extensively documented in the past. To provide a broad characterization of the urinary volatile compounds (potential chemosignals), the urine samples from adult and young animals of both sexes, as well as from castrated animals, were examined. The volatiles were extracted through a dynamic headspace technique and analyzed by combined capillary gas chromatography–mass spectrometry. The major constituents have been identified as various ketones, aldehydes, pyrazines, pyridines, quinolines, a thiazole, an amide, and a nitrile. While no chromatographic peaks were found to be unique to gender or age, quantitative analyses revealed that numerous compounds exhibited significant variations among the different groups. Their endocrine dependencies suggest a potential chemosignaling function.

Key Words – Pheromone candidates, deermouse, chemical communication, gas chromatography/mass spectrometry.

INTRODUCTION

Both reproduction and social activity in rodents are regulated in part by chemosignals. Since the late 1950s, intensive research on the pheromone communication in the house mouse (*Mus domesticus*) has implicated urine as an important source of pheromones causing estrus induction (Marsden and Bronson, 1964), puberty acceleration (Vandenbergh, 1977), and puberty delay (Drickamer, 1973). Our laboratory has followed these reports through the development

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of chemical profiling procedures that demonstrated that some, but not all urinary volatile constituents are under endocrinological control (Schwende et al., 1986; Jemiolo et al., 1987). This preselection of candidate substances has further resulted in successful characterization of several pheromones. The male-originated 2-*sec*-butyl-dihydrothiazole and dehydro-*exo*-brevicomin were active in eliciting intermale aggression (Novotny et al., 1985a), attractiveness to females (Jemiolo et al., 1985), and estrus synchronization (Jemiolo et al., 1986). The major volatile sesquiterpenes from the male preputial gland, α- and β-farnesene, appear to cause male aversion (Novotny et al., 1990) and attract females (Jemiolo et al., 1991). These compounds are also effective in estrus induction (Ma et al., 1999). 2,5-Dimethylpyrazine, a characteristic urinary volatile compound of grouped adult females, delays sexual maturation in young female mice (Novotny et al., 1985b).

Numerous biological investigations in the deermouse (*Peromyscus maniculatus*) have shown that there are at least superficial similarities between this species and *M. domesticus* in the major use of chemical communication. For example, urine from intact male deermice, but not from castrated males, contains a puberty-acceleration chemosignal (Lombardi and Whitsett, 1980; Teague and Bradley, 1978). The inhibitory effects of all-female grouping on the estrous cycle and the estrus-inducing effect of the male urine have been documented (Bronson and Dezell, 1968; Bronson and Marsden, 1964). The urinary odors of a strange male appear to inhibit functional development of corpora lutea, thus blocking implantation and returning the previously inseminated females to estrus (Bronson and Eleftheriou, 1963; Eleftheriou et al., 1962; Bronson et al., 1969). While the sexual maturation of young male deermice is retarded by a male urinary pheromone (Bediz and Whitsett, 1979; Lawton and Whitsett, 1979), the puberty of young female deermice is inhibited by signals originating from urine of adult female conspecifics (Lombardi and Terman, 1980; Lombardi and Whitsett, 1980).

To establish basic chemical information on chemosignaling in *P. maniculatus*, we analyzed the volatile constituents of urine from adult and young animals of both genders as well as castrated males. We report here the urinary substance identification and quantitation results obtained through combined gas chromatography–mass spectrometry. Statistical evaluation of the data sets points to a potential significance of certain profile constituents in chemical communication.

METHODS AND MATERIALS

Animals and Sample Collection. Deermice were bred from a stock originally purchased from the Peromyscus Stock Center, University of South Carolina, Columbia, South Carolina. Adult animals were housed singly in opaque

plastic cages and maintained with a 12-hr light–12-hr dark cycle. Formulated food (Purina Mills, Richmond, Indiana) and water were supplied ad libitum. Bedding material was changed weekly. The urine samples were collected from 12 adult males, 11 adult females, 6 castrated males, 25 immature males, and 20 immature females. The urine from juvenile animals was pooled to form four samples in each male and female group. The adult animals were about 4 months old, while the young animals were 10–17 days old. Castration was performed one month prior to sample collection. Urine was collected in the morning by holding the animal over a glass plate and gently pressing the abdominal flank. Urine was then recovered with a microsyringe, and the samples were immediately frozen and stored at −20°C until analyzed.

Sample Preparation. Urinary volatile compounds were extracted from individual samples through a dynamic headspace technique (Novotny et al., 1974), employing a porous polymer, Tenax GC (Applied Science Laboratories, State College, Pennsylvania), as the collection medium. The volatiles were sparged from 1-ml samples at 50°C with purified helium gas at 70 ml/min for 30 min. The vapor was passed through a chilled water condenser before entering the precolumn packed wtih 4.0 mg Tenax GC. To increase concentrations of the volatiles in the vapor phase, 200 mg $(NH_4)_2SO_4$ were added to each sample before extraction.

Gas Chromatography–Mass Spectrometry. The volatiles trapped on the precolumn were subsequently desorbed in the heated injection port (250°C) of a GC-MS (Finnigan MAT Magnum) instrument (Finnigan, San Jose, California) and retrapped into a liquid-nitrogen-cooled section of a capillary column. The analytical column was DB-5, 30 m × 0.25 mm ID (J&W Scientific, Folsom, California). Helium was used as the carrier gas at an inlet pressure of 12 psi. The oven temperature was maintained at 30°C for 5 min and then programmed to 210°C at 2°C/min. The transfer line temperature was set at 290°C.

Electrophoresis. Urinary proteins were isolated by size exclusion chromatography (Sephadex G-50, Pharmacia, Uppsala, Sweden) on a 60-cm × 8-mm-ID column. The isolated proteins were separated by electrophoresis on 12.5% polyacrylamide gel containing 0.1% SDS. Gels were stained with Coomassie brilliant blue and fixed with 7% acetic acid (Szoka et al., 1980; Hames and Richwood, 1990).

Mass Spectrometry of Deermouse Urinary Proteins. Cytochrome *c* (0.5 μl, 1 μM internal standard) and 0.5 μl deermouse urinary protein solutions were mixed with 1 μl sinapinic acid as the MALDI matrix. The sample was analyzed on a Voyager-DE RP BioSpectrometry workstation (PerSeptive Biosystems, Framingham, Massachusetts), equipped with delayed ion extraction capability. The nitrogen laser was used for sample desorption. The spectrum was smoothed with the 19-point method of Savitzky and Golay (1964).

Statistical Analysis. Statistical evaluation of the concentrations of volatile

compounds was performed by one-way analysis of variance with comparisons by the Tukey test. To overcome the inequality of variance, the original peak values were log-transformed prior to analysis (Zar, 1984).

RESULTS

Figure 1 shows a representative chromatogram obtained from an intact male urine sample. The peak numbers refer to the list of compounds identified in Table 1. Thirty-nine major constituents have been identified tentatively as ketones, pyrazines, pyridines, aldehydes, quinolines, a thiazole, an amide, and a nitrile. One striking feature of this volatile profile is a relatively high number of ketones (29 of 39 constituents). Although certain ketones are the expected end products of a mammalian metabolism through β- and ω-oxidation of their respective fatty acids, the number of structures observed here is unusually high. It is also interesting to note that acetophenone and ethylpyrazine were found as the dominant constituents in all samples. These two peaks are much larger in size than others.

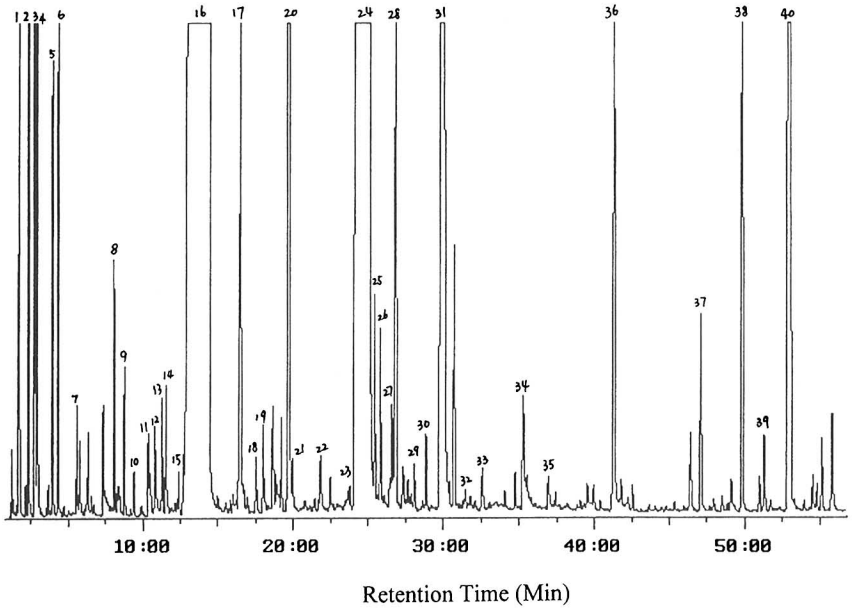

FIG. 1. Total ion chromatogram of the urinary volatiles of male deermice. The numbers refer to compound identification in Table 1. Peak 40 is the internal standard (dihexyl ketone).

TABLE 1. PEAK IDENTIFICATION AND CONCENTRATION (MEAN ± SEM) OF COMPOUNDS FROM *Peromyscus maniculatus* OF DIFFERENT STATUS[a]

Peak	Identification	Adult male (a)		Adult female (b)		Castrated male (c)		Young male (d)		Young female (e)	
1	Butanone[b]	8.95	0.14	7.20	0.30a	8.30	0.24b	7.27	0.55	7.02	0.29a
2	3-Methyl-2-butanone[b]	8.97	0.19	7.86	0.24a	7.91	0.34a	7.71	0.09	6.45	0.09a
3	2-Pentanone	8.36	0.14	8.00	0.25	8.54	0.46	9.11	0.18	8.80	0.04
4	3-Pentanone	10.67	0.23	10.16	0.20	9.81	0.55	10.75	0.18	9.98	0.12
5	4-Methyl-2-pentanone[b]	7.61	0.12	7.07	0.31	7.40	0.18	6.89	0.15	5.76	0.04ac
6	2-Methyl-3-pentanone[b]	7.88	0.13	6.22	0.29a	6.89	0.26a	6.36	0.14	4.25	0.21abcd
7	3-Hexanone	5.93	0.15	5.68	0.32	5.37	0.31	6.00	0.29	5.59	0.11
8	4-Methyl-3-penten-2-one	6.50	0.13	4.42	0.25a	5.35	0.27ab	5.70	0.26	5.02	0.06a
9	*trans*-2-Hexenal	6.23	0.11	6.64	0.48	6.15	0.15	6.51	0.13	5.66	0.47
10	5-Methyl-2-hexanone[b]	5.29	0.25	1.54	0.51a	4.31	0.20b	0.00		3.80	0.11
11	2-Heptanone	6.08	0.18	6.01	0.38	6.01	0.25	6.93	0.53	6.32	0.21
12	2,6-Dimethylpyridine[b]	6.13	0.14	6.16	0.55	5.47	0.26	6.74	0.16	5.69	0.05
13	3-Heptanone[b]	6.09	0.13	5.91	0.34	5.55	0.22	6.29	0.40	5.86	0.06
14	2-Heptanone[b]	6.26	0.15	6.48	0.36	6.37	0.13	5.67	0.59	4.55	0.69abc
15	Heptanal	4.91	0.51	3.76	0.59	3.41	0.87	5.41	0.32	5.84	0.15
16	Ethylpyrazine[b]	13.39	0.23	14.61	0.30a	12.18	0.26ab	10.48	0.28b	11.79	0.50b
17	Benzaldehyde[b]	7.30	0.16	7.51	0.25	7.17	0.34	8.54	0.10	8.38	0.08
18	4-Cycloheptanone	5.51	0.12	4.51	0.46	4.85	0.33	5.88	0.48	6.06	0.07
19	6-Methyl-5-heptenal	5.98	0.14	5.38	0.39	4.63	0.33	5.67	0.16	5.57	0.06
20	2,3-Ethylmethylpyrazine[b]	8.42	0.24	9.42	0.31	7.77	0.34b	6.97	0.35b	7.56	0.18b
21	Octanal	5.09	0.55	3.25	0.84	4.70	1.18	5.98	0.14	5.92	1.16

TABLE 1. CONTINUED

Peak	Identification	Adult male (a)		Adult female (b)		Castrated male (c)		Young male (d)		Young female (e)	
22	2,4,6-Trimethylpyridine	4.55	0.69	4.34	0.35	4.89	0.60	4.00	0.60	4.90	0.02
23	6-Methyl-2-octanone	4.31	0.18	2.09	0.71a	4.11	0.18b	0.00		0.00	
24	Acetophenone	12.64	0.13	12.42	0.34	11.98	0.18	11.32	0.09	10.92	0.12a
25	An ethyldimethylpyrazine	5.90	0.71	6.97	0.31	4.15	1.06	0.00		5.18	0.17
26	An ethyldimethylpyrazine	6.13	0.68	6.83	0.33	4.52	1.16	0.00		5.05	0.04
27	Propiophenone[b]	5.90	0.22	6.61	0.39	5.76	0.22b	4.99	1.97ab	5.99	0.00
28	Unknown	6.82	0.35	7.73	0.33	5.77	0.31	4.00	0.60	6.24	0.27
29	A methylacetophenone	4.59	0.76	4.51	0.64	4.50	0.64	6.34	0.68	6.49	0.55
30	2-Methylacetophenone	6.50	0.17	6.49	0.40	6.30	0.13	5.96	0.31	5.44	0.18
31	Phenylacetonitrile	9.42	0.25	9.93	0.24	8.34	0.59b	7.54	0.40b	8.06	0.20
32	6-Methyl-2-nonanone	2.07	0.61	0.69	0.48	2.94	0.79	2.65	0.35	5.55	0.50b
33	Naphthalene[b,c]	5.22	0.78	5.50	0.45	5.20	0.16	5.99	0.72	5.11	1.42
34	Formanilide	6.51	0.75	6.34	0.47	7.15	0.66	7.14	0.04	6.92	0.17
35	Benzothiazole[b]	3.55	0.71	4.80	0.73	5.77	0.19	0.00		0.00	
36	2-Methylquinoline[b]	7.02	0.72	4.93	0.67	6.13	0.43	0.00		0.00	
37	A dimethylnaphthalene[c]	6.10	0.26	2.74	0.86a	4.29	1.16	0.00		0.00	
38	A methylethylquinoline	7.09	0.28	5.36	0.73	5.22	0.92	0.00		0.00	
39	Geranylactone	5.24	0.58	5.34	0.47	4.88	0.19	6.24	0.52	6.30	0.06

[a] The adult male, adult female, castrate male, young male and young female groups are represented by a, b, c, d, e, respectively. If a group is marked with a letter from another group, the difference between the two groups are judged significant, $P < 0.05$).

[b] Compound structures were confirmed by mass spectra and retention times of authentic chemicals.

[c] These are most likely contaminants.

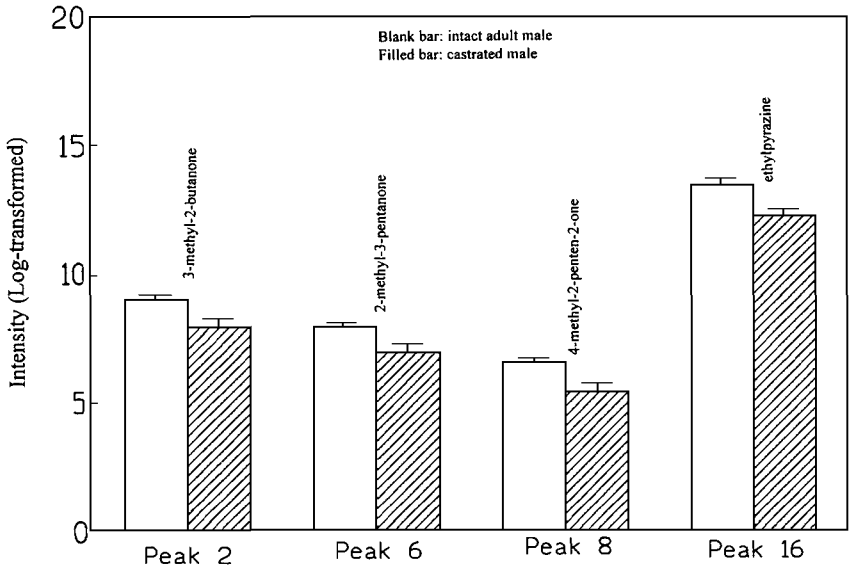

FIG. 2. Quantitative comparison between the profiles of the adult and castrated males. Differences for the selected compounds are significant at $P < 0.05$.

Visual examinations of all chromatograms indicated that there were no consistent qualitative differences between the profiles of adult and castrated males, i.e., no major peaks were missing in either set. However, through the statistical analyses, we established that four compounds, 3-methyl-2-butanone, 2-methyl-3-pentanone, 4-methyl-3-penten-2-one, and ethylpyrazine, displayed significant variations in their peak areas (Figure 2). Castration significantly depressed their concentrations in the urine ($P < 0.05$). For some other compounds, with the exception of peaks 3, 14, 32, 34, and 35, there was a tendency for the urine of the intact animals to contain higher concentrations than the urine of the castrated group. However, such differences were not statistically significant ($P > 0.05$). One possible reason for this may be a relatively small sample set.

We found that chromatographic profiles between adult males and females were quite similar. No sex-specific compounds could be detected in either set. However, quantitative analyses (peak areas) suggested that seven compounds showed marked differences between the two sexes (Figure 3). While the levels of six compounds (butanone, 3-methyl-2-butanone, 2-methyl-3-pentanone, 4-methyl-3-penten-2-one, 5-methyl-2-hexanone, and 6-methyl-2-octanone) were elevated in the adult male group, ethylpyrazine exhibited a stronger presence in the adult female group.

Both qualitative and quantitative differences were detected between the

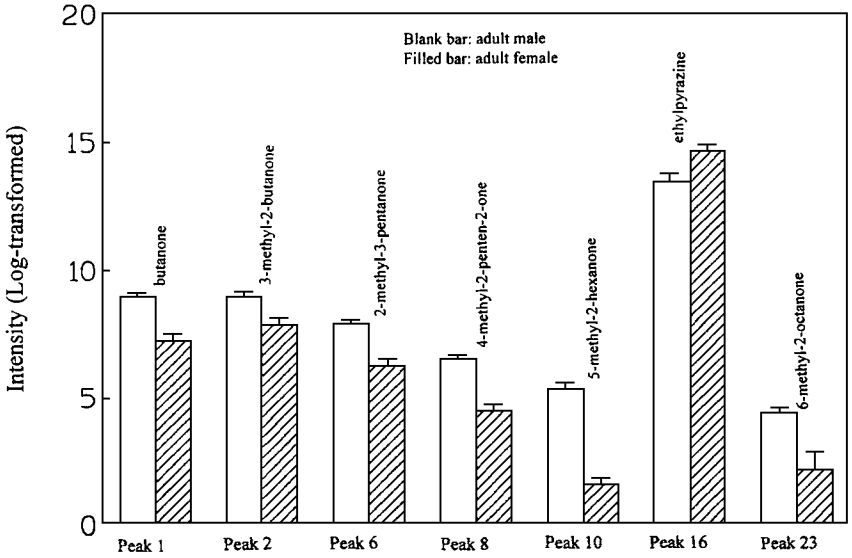

FIG. 3. Quantitative comparison between the profiles of the adult males and females. Differences for the selected compounds are significant at $P < 0.05$.

profiles of adult and juvenile animals. Comparisons between the adult male group and the young male group indicated that only one compound, propiophenone, showed a higher concentration in the adult males compared to the young animals. However, seven peaks [5-methyl-2-hexanone, 6-methyl-2-octanone, two ethyldimethylpyrazines (peaks 25, 26), benzothiazole, 2-methylquinoline, and a methylethylquinoline (peak 28)] were totally missing from the GC profiles of young males (Figure 4). Changes in the nature and abundance of volatiles also occurred when young females became mature. While five peaks (2-methyl-3-pentanone, 2-heptanone, ethylpyrazine, 2,3-ethylmethylpyrazine, and 6-methyl-2-nonanone) showed marked differences between the two sample types, five compounds [6-methyl-2-octanone, benzothiazole, 2-methylquinoline, and a methyl, and ethylquinoline (peak 38)] were totally missing in the profiles of young females (Figure 5).

DISCUSSION

The primary focus of this investigation has been a broad chemical characterization of volatile urinary constituents in the deermouse. In contrast to the previously characterized house mouse urinary volatiles (Schwende et al., 1986), fewer constituents were encountered in this species. While the house mouse contains

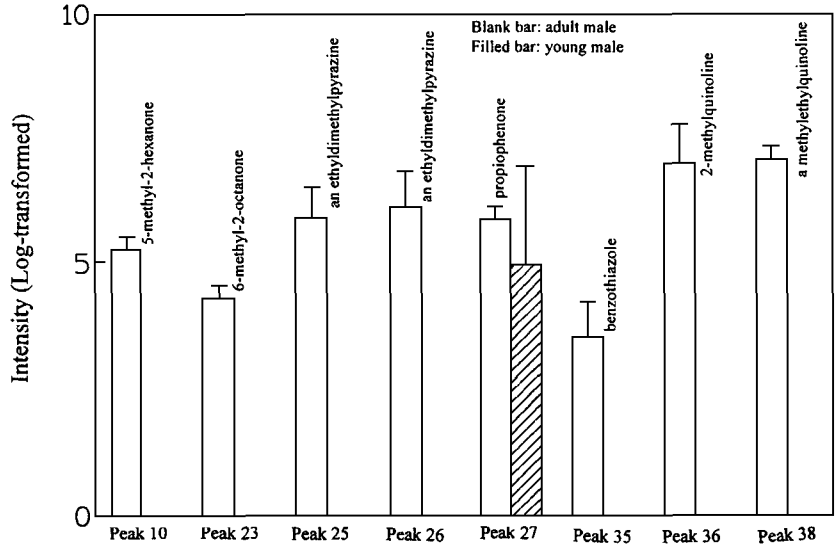

FIG. 4. Quantitative comparison between profiles of the adult and young males. Differences for the selected compounds are significant at $P < 0.05$.

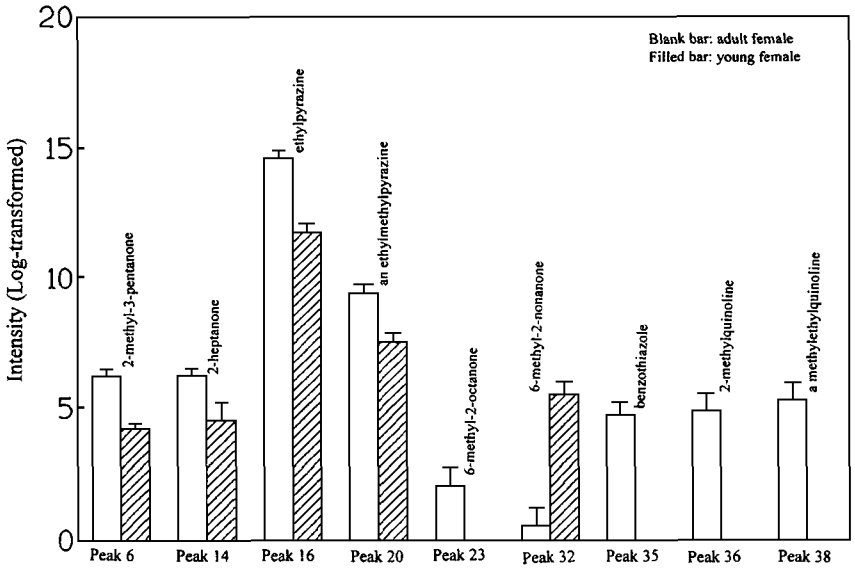

FIG. 5. Quantitative comparison between the profiles of the adult and young females. Differences for the selected compounds are significant at $P < 0.05$.

a wider repertoire of chemicals (well over 100), less than 40 detectable peaks were seen in the deermouse, with most featuring a chemical makeup different from the house mouse. The house mouse features a set of unique, male-specific compounds, such as 2-*sec*-butyl-dihydrothiazole, dehydro-*exo*-brevicomin, and α- and β-farnesene, which have been implicated as distinct chemosignals, both endocrinologically and behaviorally. In contrast, we have found here no substances that would distinguish male from female deermice.

Yet another distinguishing characteristic of house mouse urine is excretion of a large amount (typically, 1–5 mg/ml) of a proteinaceous material, the so-called "major urinary protein" (MUP) (Finlayson et al., 1963). The MUP is now known to be a conglomerate of protein isoforms (Szoka et al., 1980) with the molecular mass of approximately 17,800 Da. The excretion of MUP is androgen-dependent (Clissold et al. 1984; Szoka and Paigen, 1978), as female house mice produce only a small fraction of urinary protein, but the biosynthesis becomes stimulated substantially with testosterone treatment. Interestingly, all volatiles with a distinct male-to-female pheromonal activity bind strongly to MUP (Novotny et al., 1998), supporting the general notion that the MUP complex, or some of its components, may function as the carriers or reservoirs of pheromones. Likewise, a similar androgen-dependent urinary protein with a molecular weight of 16.7 kDa was also determined in the deermouse urine (Figure 6), but no compounds were found to bind specifically to this protein.

M. domesticus and *P. maniculatus* may sometimes share the same habitat but maintain their separate colonies. A general mechanism of the male-to-female chemical communication may also be similar in both species, as evidenced by the results on puberty acceleration and estrus induction (Teague and Bradley, 1978; Bronson and Dezell, 1968; Bronson and Marsden, 1964). However, as suggested by the work presented here, they seem to feature entirely different sets of urinary volatiles.

Several volatile compounds found in the urine of *Peromyscus* (3-methyl-2-butanone, 2-methyl-3-pentanone, 4-methyl-2-pentene-2-one, and ethylpyrazine) appear androgen-dependent. This is reflected in the changes of their abundance after castration in the male and also in the measured differences between adult males and females. While these compounds could have some role in chemosignaling of the endocrine status, there is no clear-cut situation here with the substantial concentration differences that was so helpful in the previous verification of the house mouse pheromones. Alternatively, chemosignals detectable beyond the present methodology may still exist.

In the house mouse, the urine from grouped adult females delays sexual maturation of young females (Drickamer, 1977) and inhibits a normal estrous cycle in adult animals (Ma et al., 1998). 2,5-Dimethylpyrazine is a unique constituent, being primarily responsible for the delay of puberty (Novotny et al., 1985b) and estrus suppression (Jemiolo and Novotny, 1993).

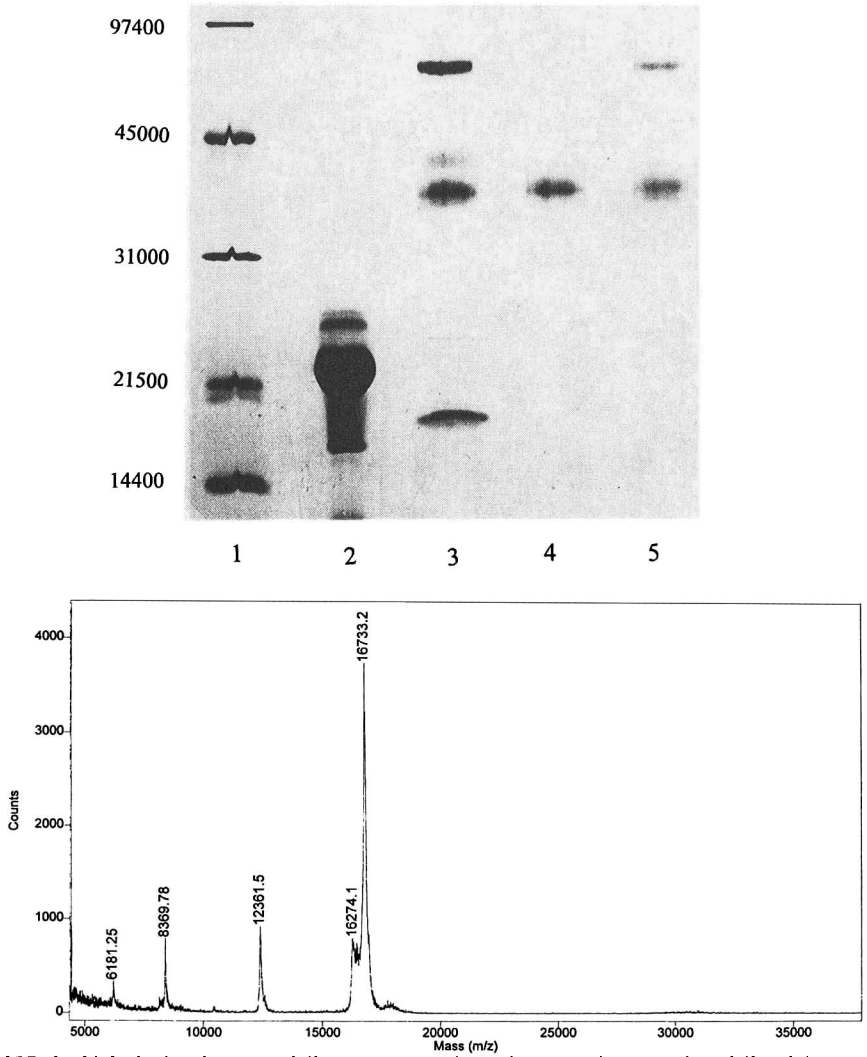

FIG. 6. Gel electrophoresis of the urinary proteins (comparative runs) and the determination through mass spectrometry. Top: proteins separated by electrophoresis on 12.5% polyacrylamide gel containing 0.1% molecular weight standards; lane 2, male house mouse MUP; lane 3, male deermouse urinary proteins; lane 4, female deermouse urinary proteins; lane 5, castrated male deermouse primary proteins. Botton: matrix-assisted laser desorption/ionization mass spectrum of the lower protein spot (lane 3, above) from the male deermouse. The peak with m/z of 12,361.5 Da is due to cytochrome c (used as calibration standard). The lower-mass peaks correspond to the doubly charged ions of both proteins.

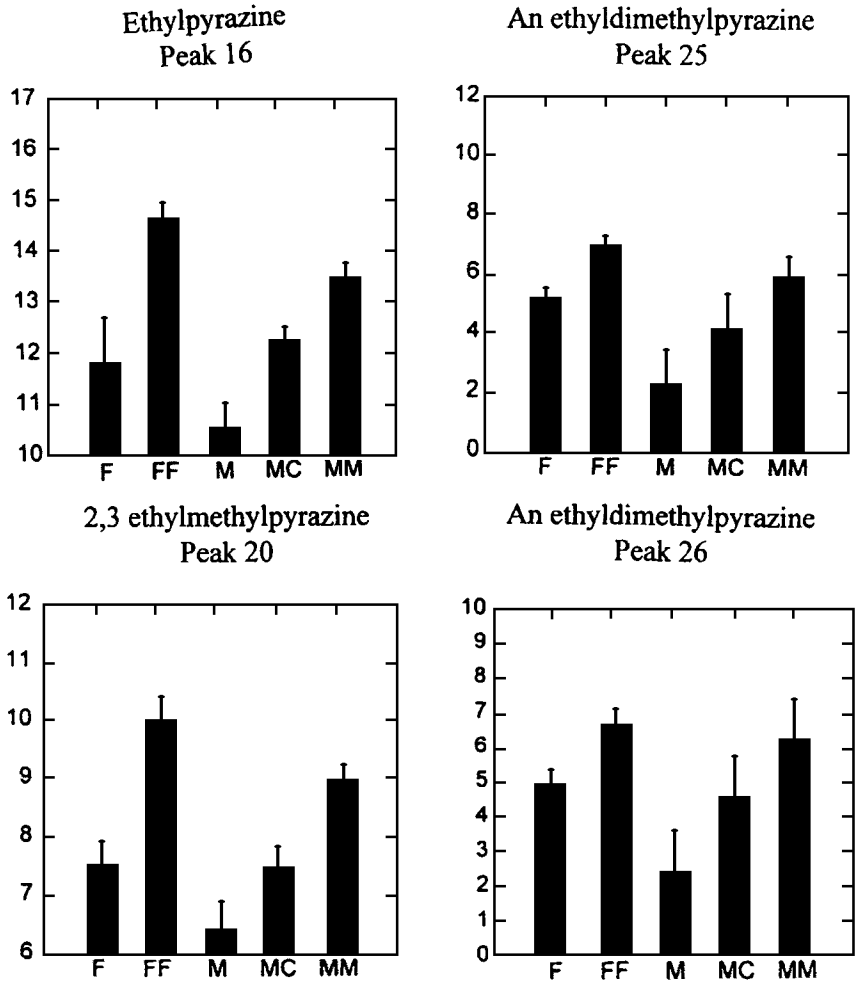

FIG. 7. Comparison of several pyrazine derivatives across the five experimental groups. F, FF, M, MC, and MM represent young female, adult female, young male, castrated male, and adult male, respectively.

Interestingly, several pyrazine derivatives are distinct urinary constituents in both male and female deermice. Ethylpyrazine, 2,3-ethylmethylpyrazine, and two ethyldimethylpyrazines also exhibit some endocrinological dependencies, as shown in Figure 7, with the adult females demonstrating the highest levels of all pyrazines. They represent a most interesting system for future biological testing in females, because pyrazines are known to be among the most odorifer-

ous substances. Our future efforst will concentrate on determination of the alkyl substituent position within these tentatively assigned pyrazines structures.

Benzaldehyde (peak 17) is a ubiquitous constituent of mammalian urine. It was previously reported in the house mouse (Schwende et al., 1986), California mouse (Jemiolo et al., 1994), pine vole (Boyer et al., 1989), wolf (Raymer et al., 1986), and the red fox (Jorgenson et al., 1978). Estradiol stimulated the formation of benzaldehyde in the wolf (Raymer et al., 1986). Benzaldehyde has also been implicated as an attraction and aggregation pheromone (Price et al., 1994) in the insect world. In this work, however, benzaldehyde has not shown any endocrinological dependence in deermice.

Significant variations were observed between the juvenile and adult animals, demonstrating an additional role of the hormonal system in the excretion of urinary secondary metabolites. Certain distinctly odoriferous volatiles were not represented in the profiles of juvenile animals; in addition to the already discussed series of ketones and pyrazines (undetectable in young males), benzothiazole and alkylquinolines were most noticeably absent in all juveniles.

CONCLUSIONS

We have presented here a broad chemical characterization of the urinary volatile constituents of *P. maniculatus*. Although no unique volatiles were found in relation to gender differences, numerous structurally interesting compounds (variously substituted ketones, pyrazines, pyridine, derivatives, benzothiazole, and alkylquinolines) appear to be under endocrinological control because their levels vary with sex, age, and the presence of endocrine glands. Deermice apparently excrete urinary volatiles that are chemically different from those in house mice. This information provides the groundwork for future biological testing.

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IDENTIFICATION OF SEMIOCHEMICALS ASSOCIATED WITH JEFFREY PINE BEETLE, *Dendroctonus jeffreyi*

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Abstract—Analysis of frass extracts and aerations from adult beetles confirmed earlier studies identifying 1-heptanol as a principal pheromone constituent produced by female *Dendroctonus jeffreyi* Hopkins. The aerations also indicated that males produced 33% (+) and 67% (−)-frontalin, previously unreported for this species, and >99% (+)-*exo*-brevicomin. Both males and females were responsive to these compounds in electrophysiological studies. Laboratory bioassays and field trapping experiments indicated that males and females were attracted to a mixture of 1-heptanol plus the principal host resin component heptane, but females were less attracted than males. Inclusion of racemic *exo*-brevicomin at 0.1% concentration in the traps increased the response of females. Inclusion of racemic frontalin above a 1% concentration in the normally attractive lures blocked arrival of beetles to the traps. While the female-produced pheromone components and host synergists are very different between the sibling species *D. jeffreyi* and *Dendroctonus ponderosae* Hopkins, the principal male-produced compounds are the same, and the response of the female beetles to those compounds appears to be similar.

Key Words—*Dendroctonus jeffreyi*, *Pinus jeffreyi*, heptane, heptanol, *exo*-brevicomin, frontalin, semiochemical, pheromone.

INTRODUCTION

Primary bark beetles in the genus *Dendroctonus* that are near-obligate parasites (Raffa et al., 1993) colonize living healthy trees, and the trees die as a consequence of mass colonization (Coulson, 1979). The colonization process is medi-

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ated by semiochemicals released by attacking females and the responding males (D. L. Wood, 1982; Borden et al., 1987). Raffa and Berryman (1983) suggested attractant pheromones will be produced as long as the tree is actively resisting, and production is terminated when resistance has been overcome. The combination of reduced production of aggregation pheromones and release of antiaggregation pheromones by beetles or the autoxidation of host resin components to inhibitory compounds deters continued arrival on the host, ends the colonization process, and limits competition among colonizing beetles (D. L. Wood, 1982; Borden et al., 1987; Hunt et al., 1989).

Dendroctonus jeffreyi Hopkins, the Jeffrey pine beetle, and *D. ponderosae* Hopkins, the mountain pine beetle, are sibling species in western North America (S. L. Wood, 1982). *Dendroctonus ponderosae* colonizes up to 13 different species of pines and its geographic range completely overlaps the distribution of *D. jeffreyi*. *Dendroctonus jeffreyi* colonizes only *Pinus jeffreyi* Grev. & Balf., which is not a host for *D. ponderosae*. The volatile components of the oleoresin of host pines of the mountain pine beetles are primarily monoterpenes; however, the composition of *P. jeffreyi* oleoresin is approximately 90% *n*-heptane (Mirov, 1961; Savage et al., 1996a,b).

Pitman et al. (1969) analyzed the volatile components of hindguts of both sexes of *D. jeffreyi* and identified *cis*- and *trans*-verbenol, verbenone, and brevicomin (*endo*- or *exo*-7-ethyl-5-methyl-6,8-dioxobicyclo[3.2.1]octane) from females and *trans*-verbenol plus brevicomin from males. Subsequently, Renwick and Pitman (1979) extracted the hindguts of male and female *D. jeffreyi* and identified 1-heptanol and 2-heptanol as major components from females and minor components from males. Field bioassays conducted as part of the same study indicated that 1-heptanol was an attractant for both sexes, and suggested that 2-heptanol could inhibit aggregation.

It has become clear in the years since the pheromone of *D. ponderosae* was initially described as a single female-produced compound, *trans*-verbenol (Pitman et al., 1968; Pitman and Vité, 1969; Pitman, 1971), that there are semiochemicals released by both males and females, and the colonization process is governed by the sequence and quantity of the compounds produced (Libbey et al., 1985; Borden et al., 1987). Despite the considerable amount of work that has been directed towards understanding chemical communication in the sibling species, the semiochemicals used by *D. jeffreyi* have remained poorly understood. The objective of this study was to identify the pheromone components produced by both sexes of *D. jeffreyi* and relate these compounds to the process of host colonization.

METHODS AND MATERIALS

Insects. All beetles used in the study were collected from field populations in the San Bernardino mountains (San Bernardino County, California) or

the Sierra Nevada mountains (Mono County, California). Infested *P. jeffreyi* were felled when progeny beetles were in late larval and pupal stages, cut into 1-m-long sections, and held at ca. 2°C in an environmental chamber. As adult beetles were needed, log sections were placed into emergence chambers (Browne, 1972). Emerging beetles were collected daily, separated by sex, and stored in a refrigerator at 5°C until used. Twenty-four hours prior to use, the beetles were removed from storage and placed in a 27,000-cm^3 screen cage to warm to room temperature and exercise.

Synthetic Standards. Verbenone (chemical purity > 98%, enantiomeric composition 85% −/15% +), *trans*-verbenol [chemical purity 80% (contains 12.6% *cis*-verbenol), enantiomeric composition 85% −/15% +], frontalin (chemical purity 100%, racemic enantiomeric composition), *exo*-brevicomin [chemical purity 99% (contains 0.8% *endo*-brevicomin), racemic enantiomeric composition], *endo*-brevicomin [chemical purity 96.5% (contains 1.4% *exo*-brevicomin), racemic enantiomeric composition], and ipsdienol (chemical purity 96.9%, racemic enantiomeric composition) were gifts from Pherotech Inc. (Delta, British Columbia, Canada). All other compounds [*cis*-verbenol (chemical purity 95%, racemic enantiomeric composition) and 2-heptanol (chemical purity 98%, racemic enantiomeric composition)] were obtained from Aldrich Chemical (Milwaukee, Wisconsin).

Analysis of Host Volatiles. Volatiles from *P. jeffreyi* inner and outer bark, sapwood, and heartwood were qualitatively analyzed by purge-and-trap analysis, with a Tekmar (Cincinnati, Ohio) LSC 2000 purge-and-trap analyzer fitted with a Carbopak B collector, interfaced to a coupled Hewlett-Packard (Avondale, Pennsylvania) 5890 gas chromatograph-5970B mass selective detector. Volatiles were purged from chipped 5-g samples held at room temperature at a flow rate of 50 ml/min for 12 min with He, followed by a 5-min dry purge. Collected volatiles were desorbed off the collector at 240°C and directly transfered into the GC injection port through a fused silica transfer line maintained at 125°C. The cryogenically cooled GC was fitted with a Hewlett-Packard Ultra-2 column (20 m × 0.2 mm ID), programmed from 0°C/2 min, then 10°C/min to 250°C (He carrier gas at 100 kPa head pressure). Identifications were confirmed by matching retention times and mass spectra with those of standards.

Collection of Frass and Collection of Volatiles from Previously Fed Beetles and Frass. Frass was obtained by individually introducing newly emerged beetles into fresh *P. jeffreyi* logs through holes slightly larger than the diameter of the beetles drilled through the outer bark. The holes were covered with gelatin capsules that served to prevent the escape of the beetle and to collect the frass pushed from the entry hole. Each sex was introduced into separate logs. In a third set of logs, females were introduced into the log and males were introduced into the same holes 24 hr later. Frass was collected daily and stored at −70°C until used in bioassays or extractions.

Volatiles produced by adults were collected for bioassay and analysis as described by Gries et al. (1992). Newly emerged, sexed beetles were fed on fresh *P. jeffreyi* phloem for 72–96 hr, and then placed into glass tubes (4 mm ID), with each individual separated from the next by a glass bead to prevent the beetles from injuring each other. Charcoal-filtered air (100 ml/min) was passed through the tubes for 72 hr and volatiles were trapped on a bed of Porapak Q (4 g). The chemical trap was then Soxhlet extracted with pentane through a minimum of five cycles. Volatiles from frass of newly emerged female beetles (10 g aerated 115 hr at 300 ml/min) were also trapped on Porapak Q.

Fractionation of Frass Extracts. The extract of Porapak-trapped volatiles from frass was concentrated under a gentle stream of N_2 to ~100 μl, then loaded onto a prepacked column (4.5 cm × 6 mm ID) of silica gel (230–400 mesh, packed with pentane). The extract was rinsed onto the column with two 100-μl aliquots of pentane. The column was then eluted with 4 ml each of pentane, 5% ether in pentane, and ether, collecting each eluate as a separate fraction.

Analysis of Beetle Volatiles. Extracts and fractions were analyzed by coupled gas chromatography–mass spectrometry (GC-MS), with a Hewlett-Packard 5890 GC interfaced to an Hewlett-Packard 5970B mass selective detector. Compounds were identified by comparison of mass spectra and GC retention times with those of standards. Extracts were initially analyzed by a program of 50°C/3 min, then 10°C/min to 250°C (He carrier gas at 100 kPa head pressure). Later analyses were run with a program of 15°C/1 min, then 15°C/min to 45°C/0 min, and 10°C/min to 250°C (He carrier gas at 100 kPa head pressure), in order to separate the heptane peak from the solvent front. In cases where possible ambiguity still existed (e.g., monoterpene hydrocarbons, frontalin, *exo*-brevicomin), retention time matches were confirmed on a second column (DB-WAX, 20 m × 0.32 mm ID, J&W Scientific, Folsom California), with temperature programming from 50°C/2 min, then 10°C/min to 250°C.

Determination of Enantiomeric Ratios of Frontalin and exo-Brevicomin. Analyses of extracts from aeration of male beetles by GC-MS indicated that the extracts were complex. Consequently, they were fractionated by liquid chromatography as described above for female frass extracts before being submitted to GC analysis on a chiral stationary phase column. The second fraction (5% ether in pentane) was enriched in frontalin and *exo*-brevicomin. After concentration to ~100 μl, a 1-μl aliquot of this fraction was analyzed on a 30 m × 0.25 mm ID Cyclodex B column (J&W Scientific), by using splitless injection and a temperature program of 50°C/3 min, then 2°C/min to 120°C (He carrier gas at 100 kPa head pressure). On this column, the (+) enantiomers of frontalin and *exo*-brevicomin elute before the (−) enantiomers (H. D. Pierce, Jr. and J. P. LaFontaine, personal communication). Analysis of an aliquot of the extract coinjected with racemic frontalin and *exo*-brevicomin confirmed the identities of the peaks.

Electrophysiology Studies. Beetles were prepared for electroantennograms (EAG) and coupled gas chromatography–electroantennogram detection (GC-EAD) studies by using methods modified from Wadhams (1982) and Salom et al. (1991). A beetle was placed in a tapered hole drilled into a Plexiglas block to confine its movements, with the top of the head protruding out of the hole and above the surface of the block. The antennae were spread across the block surface and held down by nylon threads. The holder was transferred to a small vise and manipulated so that the insect was directly in the airstream (300 ml/min) of the 10-mm-ID delivery tube. Glass capillary electrodes with silver wire filaments and filled with electrolyte solution (Staddon and Everton, 1980) were used to record beetle antennal responses. The reference electrode was placed into a puncture in the beetle's head made with an electrolytically sharpened 0.25-mm tungsten probe, while the recording electrode was placed directly into a pre-made puncture in the club of one of the antennae. Antennal responses were amplified with a custom-built amplifier (G. Gries, Simon Fraser University, Burnaby, British Columbia), and recorded on an Hewlett-Packard 3394a integrator.

Response to individual volatiles were determined by pipetting 1 μl of the test chemical (100 ng μl in hexane) onto a small filter paper substrate inserted into a 5-mm-ID glass tube. The solvent was allowed to evaporate for 1 min before presentation of the odor to the insect. At the 1-min mark, the tube was inserted into a timer-controlled solenoid valve, which delivered a measured puff (1-sec duration at a rate of 380 ml/min) of stimulus into the airstream passing over the antenna. Odors were presented to a beetle in random order at 2-min intervals.

Antennal responses to components of volatile mixtures also were measured by coupled GC-EAD. Test materials were injected into a Hewlett-Packard 5890 Series II GC equipped with a DB-5 column (30 m $\times$ 0.32 mm ID, program 50°C/1 min, then 10°C/min to 150°C/0 min and 15°C/min to 250°C, with He carrier gas). The column effluent was split equally to two deactivated fused silica columns (1 m lengths of 0.25 mm ID). One arm was directed to the GC's FID detector, and the other arm was directed out of the GC through a heated transfer line (250°C), with the tip of the capillary projecting into the tube delivering the airstream to the antenna (as described above). Before conducting a run, antennal response was verified with a puff of 1-heptanol vapor (100 ng/μl in hexane) introduced into the airstream via a syringe.

Materials analyzed by electroantennography included Porapak extracts of frass and of beetles, and blends of standards, including compounds known to be pheromone components in other bark beetle species. The response to 1-heptanol (100 ng/μl in hexane) was the standard against which all other stimuli were compared, and responses to other compounds were converted to a percentage of the response to the 1-heptanol standard. The percentage response data were normalized by arc sine square root transformations before analysis by PROC GLM

on SAS (SAS Institute, 1989). Differences among treatments were determined with the Student-Newman-Keuls procedure.

Laboratory Bioassays. Walking responses of beetles to frass were determined by arena bioassays modified after Hunt and Borden (1988). The beetles, placed in an exercise cage 24 hr before each test, were pre-tested for a positive phototactic response. Groups of 6–20 beetles were released in the center of a linear arena and tested for a photopositive response to a light placed at one end of the arena. Individuals that failed to show a positive phototactic response in these trials were eliminated.

In addition to the light at one end of the arena, an airstream (1.0 liter/min) containing the odor stimulus was introduced on one side of the arena in the chemical bioassays. The airstream blew across the arena at 90° to the walking path of the beetles and was extracted by vacuum from the opposite side of the chamber. A glass tube containing 10 ml of frass was used to deliver the stimulus. A positive response to the test volatiles was recorded if the beetles turned and stayed within the 10-cm-wide plume of the odor source during the 3-min test period or made contact with the source. Individuals were tested for responses to frass from galleries containing females only, males only, females and males, or ground phloem. The numbers of beetles responding to the stimuli were converted to a percentage of the total in each test. Percentages were transformed by arc sine square root transformations before analysis by PROC GLM on SAS (SAS Institute, 1989). Differences among treatments were determined by the Student-Newman-Keuls procedure.

A turntable pitfall bioassay (Hwang et al., 1989) was used to test walking responses of male and female beetles to fractions of the female frass extract. Sexed newly emerged beetles were released into the center of a 30-cm-diameter circular arena bounded by a 10-cm-high acetate barrier coated with Teflon at the top edge to prevent escape. Test stimuli (20 μl pipetted onto a piece of cotton dental wick) were placed at the bottom of six equidistantly spaced plastic vials set on a 10-cm radius from the center of the arena. The mouth of each vial (3 cm ID) was flush with the arena floor. Stimuli tested included each of the three frass extract fractions, the recombined fractions 1–3, a solvent control, and an unbaited control. A cover was placed over the arena, and the beetles were allowed to move around for 3 hr. At the end of that period, the number of beetles in each vial was counted, and the responses to each treatment were converted to percentages of the total number of responding beetles. The data were normalized by an arc sine square-root transformation, and differences among stimuli were determined by GLM procedures and Student-Newman-Keuls means separation test on SAS (SAS Institute, 1989).

Field Trials. To test the flight responses of *D. jeffreyi*, field bioassays were conducted with multiple funnel traps (Lindgren, 1983). Funnel traps were suspended from reinforcing bar supports fixed to steel fence posts that were driven

into the ground. Three lines of traps, each trap in the line separated by at least 25 m and lines separated by at least 50 m, were placed at Barton Flats (San Bernardino County, California) in a mixed-age *P. jeffreyi* stand. The number of treatment replicates within each line (1–3) varied according to the specific requirements of each experiment. Test stimuli were suspended under the cover of the trap, and traps were shifted in position systematically following daily beetle collections (Paine and Hanlon, 1991).

The semiochemical release devices were constructed by fitting a glass tube (2.5 mm ID) through a hole drilled in the screw-up of a 11.1-ml glass vial and inserting a length of pipe cleaner into the glass tube to act as a wick. The rate of release was a function of the length of wick exposed above the end of the glass tube. Release rates were calculated in preliminary laboratory trials by measuring mass loss over a specific time interval from release devices aged in a fume hood.

Initial field trials were conducted to determine the response of beetles to individual compounds, to combinations of compounds isolated from aerations, and to other known bark beetle semiochemicals. Based on the results of these initial tests, subsequent field trapping studies were conducted to determine the optimal release rate of the most attractive standard combination of female-produced chemical (1-heptanol) plus host volatile (heptane) and to determine the optimal release ratio of the two components. Differences in response to traps baited with different lures in these trials were determined by GLM procedures and the Student-Newman-Keuls means separation test on SAS (SAS Institute, 1989). These studies provided the basis for development of the standard trap lure used in subsequent studies to evaluate the behavioral effects of male-produced compounds.

The flight responses of *D. jeffreyi* to frontalin and *exo*-brevicomin were investigated in four additional field tests. Formulated racemic frontalin and *exo*-brevicomin were released in the first test from microcentrifuge tubes (release rates determined by the supplier, Pherotech Inc.). Each individual compound or both compounds together were released in combination with the standard 5% 1-heptanol in heptane released at 250 mg/24 hr (determined from the study described above). In three subsequent tests, racemic frontalin, *exo*-brevicomin, or a combination of the two (20% *exo*-brevicomin in frontalin as determined from extract analysis) were incorporated into the standard lure to test response to a range of concentrations of these compounds. Each treatment was released from a separate device as described above. The sex of the beetles collected from the traps was determined and the differences in the responses of each sex was determined by GLM procedures and Student-Newman-Keuls means separation test on SAS (SAS Institute, 1989). The data were transformed [square root (x_i + 0.5)] prior to analysis in those cases where there was a correlation between means and variances.

RESULTS

Analysis of Host Volatiles. In descending order, *n*-heptane, *p*-cymene, undecane, myrcene, estragole, nonane, and α-pinene were the most prevalent compounds detected by purge-and-trap analysis of *P. jeffreyi* heartwood. Small amounts of camphene, sabinene, β-pinene, β-phellandrene, and octane were also identified. Traces of a number of other unidentified monoterpenes, sesquiterpenes, and miscellaneous compounds were not conclusively identified. The volatiles profile of chipped sapwood was virtually identical to that of heartwood. In contrast, the outer bark produced only trace amounts of volatiles, from which heptane, ethanol, acetone, acetic acid, *p*-cymene, and estragole were identified.

Analysis of Beetle Volatiles. Comparison of GC traces of volatiles collected from fed, newly emerged beetles confined in tubes revealed that both male and female extracts contained large quantities of heptane and lesser amounts of 1-heptanol. In addition, males produced several compounds that were not seen in extracts from females. The two most significant male-specific peaks were identified as frontalin and *exo*-brevicomin (Figure 1). Several other minor peaks that were present in male but not female extracts were not identified. In addition, the male extract was checked by selected ion monitoring mass spectrometry for the presence of four male-specific compounds (*trans*-verbenol, verbenone, myrtenol, and ipsdienol) found in the closely related mountain pine beetle, *D. ponderosae*, but none of these compounds was found.

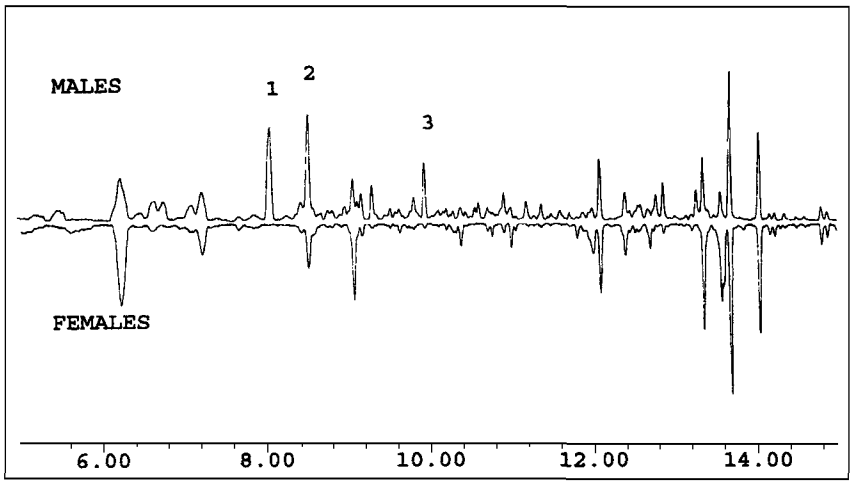

FIG. 1. Gas chromatograph–mass spectrometer traces of male and female beetle aerations (1 = frontalin, 2 = 1-heptanol, 3 = *exo*-brevicomin).

TABLE 1. RESPONSES OF NEWLY EMERGED JEFFREY PINE BEETLES TO FRASS IN NO-CHOICE LINEAR WALKING BIOASSAYS

Stimulus	Beetles responding (%), mean ± SE)[a]	
	Males (N = 6)	Females (N = 6)
Male frass	21.20 ± 6.62b	35.89 ± 12.05a
Female frass	89.64 ± 3.52a	65.44 ± 9.68a
Frass from paired beetles	15.22 ± 4.45b	45.03 ± 14.46a
Ground phloem	7.34 ± 3.45b	3.21 ± 2.21b

[a]Significant differences within a column among stimulus treatments (Student-Newman-Keuls test, male α = 0.05, df = 19; female α = 0.05, df = 19) are indicated by different lowercase letters. Replicate is the bioassay trial with 6–20 beetles tested per trial. The unbaited control treatment did not induce a response and was eliminated from the analysis.

Partial purification of a male extract by liquid chromatography followed by GC analysis of the fraction containing frontalin and *exo*-brevicomin on a chiral stationary phase column revealed that males produced 67% (−)-frontalin and > 99% (+)-*exo*-brevicomin.

Laboratory Bioassays. Frass extracts were complex and were dominated by heptane and monoterpene hydrocarbons. There were no obvious differences in the GC traces from extracts of frass from newly emerged males or females. However, bioassay of the frass clearly showed that the frass produced by newly emerged females was attractive to both males (F = 39.54, df = 3, P = 0.0001) and females (F = 7.54, df = 3, P = 0.0016) (Table 1). In contrast, frass produced by males or paired males plus females was significantly attractive to females but not to males. Fresh phloem tissue was not attractive to either sex.

Fractionation of the female frass extract by liquid chromatography produced three fractions containing, respectively, hydrocarbons, weakly polar compounds such as ketals and carbonyl compounds, and moderately polar compounds including alcohols. Multiple choice bioassays revealed that fraction 3 was the most attractive fraction to both male (F = 6.06, df = 5, P = 0.0019) and female (F = 8.19, df = 5, P = 0.0001) beetles, and that fraction 3 was as attractive to both sexes as the recombined fractions (Table 2). GC-MS analysis revealed that the major components of this fraction were 1-heptanol and 2-heptanol. All further bioassays to optimize the pheromone blend were carried out in the field.

Electrophysiology Studies. In GC-EAD studies with both male and female beetles, we used Porapak Q extracts from previously fed female or male beetle aerations as the stimulus. Antennae of both sexes responded strongly to compounds eluting at the retention times of 1-heptanol. Antennae from females responded weakly at retention times corresponding to frontalin, *exo*-brevicomin, and 1-octanol in aerations from male beetles (Figure 2A). However, the corresponding male responses to the female aerations showed that males responded

TABLE 2. RESPONSES OF NEWLY EMERGED MALE AND FEMALE JEFFREY PINE BEETLES TO LIQUID CHROMATOGRAPHY FRACTIONS OF FEMALE FRASS EXTRACT IN MULTIPLE-CHOICE, TURNTABLE BIOASSAYS

Stimulus	Beetles responding (mean ± SE)[a]	
	Males	Females
Fraction 1	2.25 ± 1.11b	2.88 ± 0.61b
Fraction 2	1.00 ± 0.41b	1.88 ± 0.40b
Fraction 3	6.25 ± 1.93a	4.75 ± 0.37a
Recombined Fractions 1–3	6.50 ± 0.65a	5.00 ± 0.71a
Solvent blank	1.00 ± 0.58b	1.88 ± 0.30b

[a]Bioassays were replicated four times with males (2 × 30, 2 × 29 beetles) and eight times with females (6 × 29 beetles, 2 × 30 beetles). Significant differences within a column among treatments (Student-Newman-Keuls test, male $\alpha = 0.05$, $df = 18$; female $\alpha = 0.05$, $df = 42$) are indicated by different lowercase letters.

strongly to 1-heptanol, present in significant amounts, and to verbenone, but at a reduced level (Figure 2B).

Additional EAG analyses with selected synthetic bark beetle pheromones suggested that antennae of both sexes respond not only to racemic frontalin and racemic *exo*-brevicomin, but also to a variety of other bark beetle pheromones (Table 3). However, there were significant differences in the responsiveness of the sexes to the compounds tested [male trial ($F = 5.23$, $df = 6$, $P = 0.0001$), female trial ($F = 6.76$, $df = 6$, $P = 0.0001$)]. Relative to the 1-heptanol standard, both sexes produced the greatest response to frontalin with significantly lower responses to *exo*-brevicomin. However, the response of females to racemic 2-heptanol was not different from their response to either frontalin or *exo*-brevicomin. The response of males to racemic 2-heptanol was significantly lower than to either frontalin or *exo*-brevicomin.

Field Trials. There were significantly more beetles attracted to combinations of prospective pheromone constituents ($F = 13.46$, $df = 8$, $P = 0.0001$) than to any individual compound in the first field test (Table 4). Neither heptane alone nor 1-heptanol alone attracted significantly more beetles than the unbaited control. However, in combination, the compounds were more attractive than the control. Addition to racemic *exo*-brevicomin or *trans*-verbenol alone or in combination did not change the response of *D. jeffreyi* to 1-heptanol plus heptane.

The second and third field trials explored optimal concentrations and release rates of an attractant that combined 1-heptanol and the principal host resin component, heptane. A release rate of 250–300 mg/24 hr of the 25% mixture of 1-heptanol in heptane was significantly more attractive ($F = 12.62$, $df = 4$, $P = 0.0001$) than any of the other release rates tested in a dosage study (Table 5). Furthermore, more beetles were caught ($F = 57.58$, $df = 4$, $P = 0.0001$) when

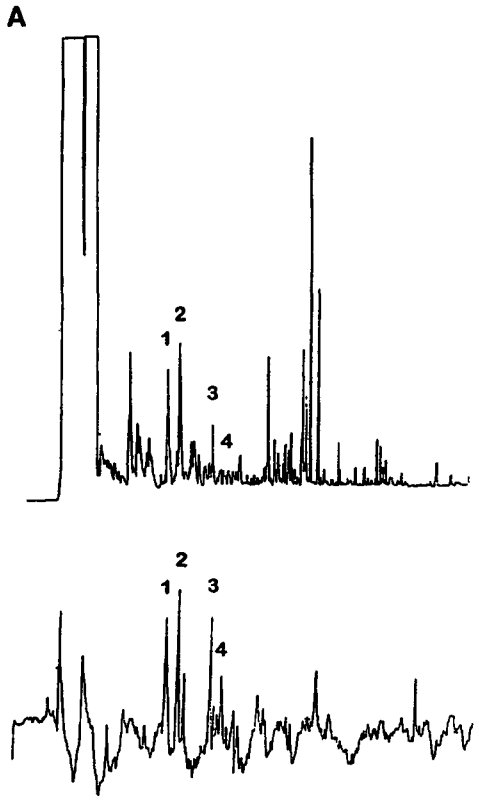

FIG. 2. Gas chromatograph–electroantennograph traces of male and female responses to aerations of previously fed beetles. (A) Gas chromatograph trace of aeration from male is shown above electroantennograph trace of female antennal response (1 = frontalin, 2 = 1-heptanol, 3 = *exo*-brevicomin, 4 = 1-octanol). (B) Gas chromatograph trace of aeration from female is shown above electroantennograph trace of male antennal response (1 = 1-heptanol, 2 = verbenone).

the release devices contained 5% 1-heptanol in heptane than were attracted to lower dilutions of the beetle-produced semiochemical (Table 6). While release of 1% 1-heptanol in heptane also attracted a significant number of beetles relative to the unbaited control, there were no differences in trap catch between the unbaited control and lower concentrations.

Other field trials explored the behavioral response to the male-produced compounds (Tables 7–10). Addition of racemic *exo*-brevicomin to the 1-heptane/heptanol standard resulted in a significant reduction ($F = 24.58$, $df = 4$, $P = 0.0001$) in the trap catch compared to the 1-heptane/heptanol lure alone, but

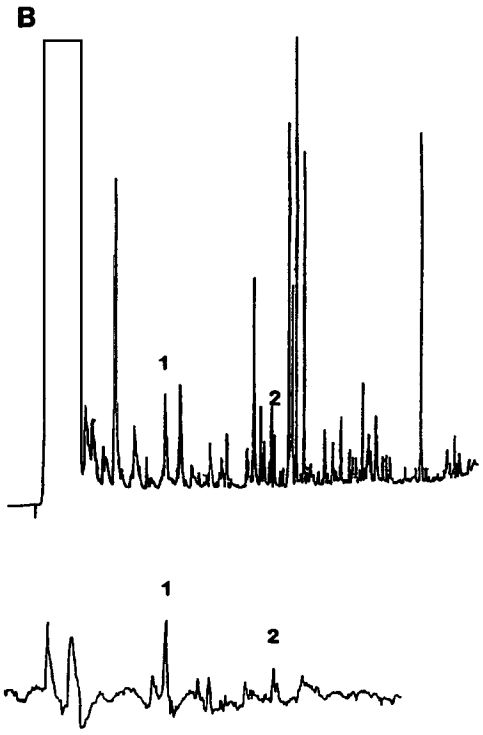

FIG. 2. Continued.

the response to this combination was still greater than to the unbaited control (Table 7). However, addition of racemic frontalin or the combination of racemic frontalin and racemic *exo*-brevicomin to the 1-heptane/heptanol lure reduced the response to the level of the unbaited control (Table 7).

Additional field studies that tested response of *D. jeffreyi* to a range of concentrations of *exo*-brevicomin and frontalin in the presence of 1-heptane/heptanol indicated that there were differences between sexes in their response (Tables 8–10). Release of racemic *exo*-brevicomin with the 1-heptane/heptanol lure elicited significant ($F = 2.84$, $df = 5$, $P = 0.03$) attraction of females at a concentration of 0.10% *exo*-brevicomin, but did not affect response to the attractant lure at any other concentration (Table 8). Inclusion of *exo*-brevicomin at any concentration had no significant ($F = 1.17$, $df = 5$, $P = 0.33$) effect on male behavior. Total trap catch was significantly ($F = 2.30$, $df = 5$, $P = 0.05$) improved over the 1-heptane/heptanol lure at only the 0.10% release rate of *exo*-brevicomin.

TABLE 3. ELECTROANTENNOGRAM RESPONSES OF MALE AND FEMALE JEFFREY PINE BEETLES TO POTENTIAL PHEROMONE COMPONENTS[a]

Compound	Response [mean ± SE (*N*)][b]	
	Male	Female
endo-Brevicomin	32.6 ± 4.51 (21)bc	29.8 ± 6.53 (14)cd
exo-Brevicomin	43.3 ± 6.65 (21)b	37.4 ± 6.68 (20)bc
Frontalin	60.5 ± 5.30 (22)a	55.7 ± 4.96 (33)a
2-Heptanol	21.3 ± 8.08 (13)c	48.6 ± 11.34 (12)ab
cis-Verbenol	40.8 ± 6.35 (19)b	18.5 ± 11.00 (9)d
trans-Verbenol	39.1 ± 7.46 (22)b	20.1 ± 27.55 (11)d
Verbenone	34.9 ± 6.09 (12)bc	34.0 ± 10.28 (11)bcd

[a]Response to 1-heptanol (100 ng/μl in hexane) used as standard and responses to test compounds presented as a percentage of the response to 1-heptanol.
[b]Significant differences within a column among treatments (Student-Newman-Keuls test, male α = 0.05, df = 92, female α = 0.05, df = 69) are indicated by different lower case letters.

Release of racemic frontalin with the 1-heptane/heptanol lure showed inhibition (F = 5.17, df = 5, P = 0.001) of females at the highest concentration (1.0% frontalin) (Table 9). As in the test of *exo*-brevicomin, there was no significant (F = 1.93, df = 5, P = 0.1008) behavioral effect on the male response by racemic frontalin. There was a significant (F = 3.44, df = 5, P = 0.008) reduction of total catch at the highest concentration of frontalin.

TABLE 4. TRAP CAPTURES OF FIELD POPULATIONS OF JEFFREY PINE BEETLES IN RESPONSE TO BLENDS OF SYNTHETIC CHEMICALS (AUGUST 1993, BARTON FLATS, SAN BERNARDINO COUNTY)[a]

Stimulus	Total beetles responding [mean ± SE (*N*)]
1-Heptanol/heptane/*exo*-brevicomin	3.59 ± 0.82 (16)a
1-Heptanol/heptane/*trans*-verbenol	3.16 ± 0.68 (16)a
1-Heptanol/heptane/*exo*-brevicomin/*trans*-verbenol	2.63 ± 0.45 (16)a
1-Heptanol/heptane	2.41 ± 0.59 (16)a
Unbaited control	0.09 ± 0.09 (16)b
Heptane	0.09 ± 0.09 (16)b
exo-Brevicomin	0.07 ± 0.07 (16)b
1-Heptanol	0.07 ± 0.07 (16)b
trans-Verbenol	0.00 ± 0.00 (16)b

[a]All chemicals released from separate devices. Release rates were 1-heptanol at 400 mg/24 hr, heptane at 250 mg/24 hr, *exo*-brevicomin at 2.4 mg/24 hr, and *trans*-verbenol at 1.3 mg/24 hr. Significant differences among treatments (Student-Newman-Keuls test, α = 0.05, df = 120) are indicated by different lowercase letters.

TABLE 5. TRAP CAPTURES OF FIELD POPULATIONS OF JEFFREY PINE BEETLE IN RESPONSE TO LURES RELEASING DIFFERENT RATES OF 25% 1-HEPTANOL DISSOLVED IN HEPTANE (JUNE 1994, BARTON FLATS, SAN BERNARDINO COUNTY)

Release rate (mg/24 hr)	Beetles caught/trap/day [mean ± SE (*N*)][a]
250–300	6.19 ± 0.94 (48)a
130–170	3.44 ± 1.10 (48)b
30–50	3.10 ± 0.69 (48)b
75–100	1.98 ± 0.60 (48)b
Unbaited control	0.02 ± 0.02 (48)c

[a]Significant differences among treatments (Student-Newman-Keuls test, $\alpha = 0.05$, $df = 210$) are indicated by different lowercase letters.

The release of both male-produced compounds (racemic frontalin and racemic *exo*-brevicomin at a 5 : 1 ratio) with the 1-heptane/heptanol lure resulted in the same basic pattern of response (Table 10). Fewer females were caught at the highest two rates of the male-produced compounds compared to the three lower release rates ($F = 5.65$, $df = 5$, $P = 0.0007$). As in the tests of response to individual compounds, there were no significant changes ($F = 1.35$, $df = 5$, $P = 0.25$) in behavioral responses of males to the lures. There was inhibition ($F = 5.16$, $df = 5$, $P = 0.0005$) of total catch at the two highest concentrations relative to the three lower concentrations, but only the response to 0.01% was significantly different from the standard lure without either male-produced compound.

TABLE 6. TRAP CAPTURES OF FIELD POPULATIONS OF JEFFREY PINE BEETLE IN RESPONSE TO LURES CONTAINING DIFFERENT COMBINATIONS OF 1-HEPTANOL DISSOLVED IN HEPTANE AT ONE RELEASE RATE (250 MG HEPTANE/DAY) (SEPTEMBER 1993, BARTON FLATS, SAN BERNARDINO COUNTY)

Stimulus (% of 1-heptanol in heptane)	Beetles caught/trap/day [mean ± SE (*N*)][a]
5.00	49.71 ± 4.81 (23)a
1.00	24.61 ± 2.44 (22)b
0.10	6.79 ± 0.99 (23)c
0.01	3.65 ± 0.84 (20)c
Unbaited control	0.08 ± 0.08 (12)c

[a]Significant differences among treatments (Student-Newman-Keuls test, $\alpha = 0.05$, $df = 84$) are indicated by different lowercase letters.

TABLE 7. TRAP CAPTURES OF FIELD POPULATIONS OF JEFFREY PINE BEETLE IN RESPONSE TO LURES RELEASING 5% 1-HEPTANOL IN HEPTANE (1-H/H) ALONE OR IN COMBINATION WITH RACEMIC *exo*-BREVICOMIN OR RACEMIC FRONTALIN (JULY 1993, BARTON FLATS, SAN BERNARDINO COUNTY)

Stimulus	Beetles caught/trap/day [mean ± SE (*N*)][a]
1-H/H	4.98 ± 0.74 (39)a
1-H/H/*exo*-brevicomin	3.21 ± 0.53 (40)b
1-H/H/*exo*-brevicomin/frontalin	0.34 ± 0.09 (40)c
1-H/H/frontalin	0.30 ± 0.09 (40)c
Unbaited control	0.00 ± 0.00 (20)c

[a]Release rates were 5% 1-heptanol in heptane at 250 mg/24 hr, *exo*-brevicomin at 4 mg/24 hr, frontalin at 10 mg/24 hr, and 20% *exo*-brevicomin in frontalin at 10 mg/24 hr. Significant differences among treatments (Student-Newman-Keuls test, $\alpha = 0.05$, $df = 155$) are indicated by different lowercase letters.

DISCUSSION

Chemical analyses of aerations from male and female *D. jeffreyi* indicate that both males and females produce 1-heptanol, thus confirming studies by Renwick and Pitman (1979). Pitman et al. (1969) also identified *cis*-verbenol, verbenone, and brevicomin (the authors did not specify whether the isolated compound was *endo*- or *exo*-brevicomin) as minor constituents and *trans*-verbenol as a major constituent of female-produced volatiles. Male-produced volatiles

TABLE 8. TRAP CAPTURES OF FIELD POPULATIONS OF JEFFREY PINE BEETLE IN RESPONSE TO LURES RELEASING 5% 1-HEPTANOL IN HEPTANE ALONE OR IN COMBINATION WITH VARIOUS CONCENTRATIONS OF RACEMIC *exo*-BREVICOMIN (OCTOBER 1993, BARTON FLATS, SAN BERNARDINO COUNTY)

exo-Brevicomin (%)	Beetles caught/trap/day [mean ± SE (*N*)]		
	Females	Males	Total
0.00	0.33 ± 0.26 (12)b	0.83 ± 0.37 (12)a	1.17 ± 0.58 (12)b
0.0001	0.83 ± 0.32 (12)ab	0.83 ± 0.24 (12)a	1.67 ± 0.50 (12)ab
0.001	1.08 ± 0.23 (12)ab	1.42 ± 0.42 (12)a	2.50 ± 0.54 (12)ab
0.01	0.80 ± 0.20 (12)ab	1.60 ± 0.43 (10)a	2.40 ± 0.52 (10)ab
0.1	1.92 ± 0.50 (12)a	1.92 ± 0.47 (12)a	3.83 ± 0.89 (12)a
1.00	1.08 ± 0.36 (12)ab	1.33 ± 0.43 (12)a	2.41 ± 0.71 (12)ab

[a]Release rate for 5% 1-heptanol in heptane was 250 mg/24 hr. Significant differences among treatments within columns (Student-Newman-Keuls test $\alpha = 0.05$, $df = 64$) are indicated by different lowercase letters.

TABLE 9. TRAP CAPTURES OF FIELD POPULATIONS OF JEFFREY PINE BEETLE IN RESPONSE TO LURES RELEASING 5% 1-HEPTANOL IN HEPTANE ALONE OR IN COMBINATION WITH VARIOUS CONCENTRATIONS OF RACEMIC FRONTALIN (OCTOBER 1993, BARTON FLATS, SAN BERNARDINO COUNTY).

Frontalin (%)	Beetles caught/trap/day [mean ± SE (*N*)]		
	Females	Males	Total
0.00	1.42 ± 0.60 (12)ab	1.42 ± 0.45 (12)a	2.83 ± 0.87 (12)a
0.0001	0.75 ± 0.30 (12)abc	1.08 ± 0.34 (12)a	1.83 ± 0.46 (12)ab
0.001	1.58 ± 0.45 (12)a	1.58 ± 0.48 (12)a	3.17 ± 0.81 (12)a
0.01	1.25 ± 0.43 (12)ab	1.00 ± 0.37 (12)a	2.25 ± 0.64 (12)ab
0.1	0.50 ± 0.19 (12)bc	0.83 ± 0.32 (12)a	1.33 ± 0.38 (12)b
1.00	0.00 ± 0.00 (11)c	0.09 ± 0.09 (11)a	0.09 ± 0.09 (11)c

[a]Release rate for 5% 1-heptanol in heptane was 250 mg/24 hr. Significant differences among treatments within columns (Student-Newman-Keuls test, $\alpha = 0.05$, $df = 65$) are indicated by different lowercase letters.

included *trans*-verbenol as a minor component and brevicomin as a major component. While they did not isolate frontalin from either sex, we found frontalin as a major volatile in male aerations. In addition, we did not find *trans*-verbenol in either sex, nor did we find *exo*-brevicomin in females. The reasons for the differences between the studies are unclear. However, it may be a reflection of the different methods employed.

TABLE 10. TRAP CAPTURES OF FIELD POPULATIONS OF JEFFREY PINE BEETLE IN RESPONSE TO LURES RELEASING 5% 1-HEPTANOL IN HEPTANE ALONE OR IN COMBINATION WITH VARIOUS CONCENTRATIONS OF RACEMIC FRONTALIN AND *exo*-BREVICOMIN (5 : 1 RATIO) (OCTOBER 1993, BARTON FLATS, SAN BERNARDINO COUNTY)

Frontalin/*exo*-brevicomin (%)	Beetles caught/trap/day [mean ± SE (*N*)]		
	Females	Males	Total
0.00	0.63 ± 0.20 (11)ab	1.27 ± 0.33 (11)a	1.91 ± 0.11 (11)bc
0.0001	1.67 ± 0.59 (12)a	1.33 ± 0.38 (12)a	3.00 ± 0.72 (12)ab
0.001	1.72 ± 1.08 (11)a	1.18 ± 0.60 (11)a	2.91 ± 1.08 (11)ab
0.01	2.00 ± 0.36 (11)a	1.09 ± 0.58 (11)a	3.09 ± 0.85 (11)a
0.1	0.17 ± 0.11 (12)b	0.83 ± 0.38 (12)a	1.00 ± 0.87 (12)c
1.00	0.00 ± 0.00 (12)b	0.08 ± 0.08 (12)a	0.08 ± 0.08 (12)c

[a]Release rate for 5% 1-heptanol in heptane was 250 mg/24 hr. Significant differences among treatments within columns (Student-Newman-Keuls test, $\alpha = 0.05$, $df = 63$) are indicated by different lowercase letters.

The populations of beetles were low when the field tests were conducted; interpretation of the low trap catch results should be made with appropriate caution. However, the field tests confirmed previous observations (Renwick and Pitman, 1979) that heptane or 1-heptanol are not attractive alone, but when released together, the combination of compounds is attractive to both sexes of *D. jeffreyi*. However, they reported a sex ratio of beetles responding to traps baited with this combination in two trials of 1 : 0.93 male–female and 1 : 1.25 male–female. Results from our field studies indicated that male response to the 1-heptanol/heptane bait was typically greater than female response.

There was a significant increase in female response to traps baited with the 1-heptanol/heptane lure and one concentration of racemic *exo*-brevicomin (0.1%) compared to the 1-heptanol/heptane lure alone. Higher and lower concentrations of *exo*-brevicomin may be inhibitory. The study was limited by use of racemic *exo*-brevicomin, the only material available to us, instead of the (+)-enantiomer produced by males. However, despite that limitation, the pheromone constituent elicits an electrophysiological response in both males and females, and it appears that females are attracted to the mixture of 1-heptanol produced by both sexes, (+)-*exo*-brevicomin produced by males (although only racemic was tested in field trials), and heptane released by the host tree.

In contrast, females are strongly inhibited by racemic frontalin. Males also appear to be inhibited, but the effect on response was not significant. However, interpretation of the field trials may be limited by the use of racemic frontalin rather than the 67% (−)-frontalin released by males. It does appear from the trapping data that the response to attraction to the 1-heptanol/heptane lure or to that lure plus *exo*-brevicomin can be eliminated by release of 1.0% racemic frontalin.

Dendroctonus jeffreyi is one of two species reported to produce 1-heptanol. Renwick et al. (1975) investigated semiochemical communication by *D. vitei* Wood in its colonization of *P. maximinoi* (=*P. tenuifolia*). Although both sexes produce 1-heptanol, the attractant appears to be frontalin produced by females (Renwick et al., 1975). Both sexes also produce *trans*-verbenol but no biological activity was reported in that study. Mirov (1961) does not list heptane as a resin constituent of this pine, but does list the sympatric *P. oaxacana* as having 21% heptane in the resin. At one time, both *P. tenuifolia* and *P. oaxacana* were considered varieties of *P. pseudostrobus*. Thus, the confusion between pheromone production and host resin constituency could be a problem of tree species identification. The production of the same semiochemical by two congeneric species colonizing hosts that have heptane-rich resin may indicate that the evolution of semiochemical production in these species is a by-product of a detoxification pathway common to both species.

The monophagous *D. jeffreyi* and the polyphagous *Dendroctonus ponderosae* are virtually identical in appearance and are considered to be sibling

species (S. L. Wood, 1982). The principal pheromone constituents of the two species appear to be different, but there are some striking similarities in the pheromone communication systems. Pitman et al. (1968) first reported that female *D. ponderosae* produce *trans*-verbenol after feeding, but not on adult emergence prior to feeding. In contrast, Gries et al. (1992) found that emergent, unfed female *D. ponderosae* released large quantities of *trans*-verbenol. Libbey et al. (1985) showed that while males also produce *trans*-verbenol, although at lower levels than females, males alone also produce *endo*- and *exo*-brevicomin and frontalin. There were also a large number of other volatiles produced by one or both sexes and the quantities depended on the feeding host, suggesting a great plasticity in the chemicals produced and indicating that some may not be semiochemicals (Libbey et al., 1985).

In many *Dendroctonus* spp., including *D. jeffreyi*, there is a synergism between the female volatiles and host resin constituents. *Dendroctonus ponderosae* colonizes at least 12 different species of pine hosts, and this seems to influence its chemical communication. Pitman and Vité (1969) indicated that host resin synergized attractiveness of *trans*-verbenol for *D. ponderosae*, and Pitman (1971) suggested that α-pinene was the most effective single monoterpene synergist for *D. ponderosae* in *P. monicolae* forests. He reported myrcene to have a moderate synergistic effect. Billings et al. (1976) found myrcene or terpinolene to be effective synergists in *P. ponderosa* stands, while Borden et al. (1983) confirmed the effectiveness of myrcene in *P. contorta* forests.

The role of *exo*-brevicomin and frontalin in the communication system of *D. ponderosae* also seems variable. *exo*-Brevicomin at low release rates has been reported to be attractive when added to the mixture of myrcene and *trans*-verbenol (Rudinsky et al., 1974; Ryker and Rudinsky, 1982). Conn et al. (1983) reported that addition of *exo*-brevicomin resulted in increased attraction of females and shifted the sex ratio of trap catch, and they suggested that, like the differences in host synergist, *exo*-brevicomin was attractive or antiaggregative in different host populations of *D. ponderosae*.

In subsequent studies in *P. contorta* stands, Libbey et al. (1985) reported *exo*-brevicomin to be neutral at low concentrations, but could not clearly determine if the compound were inhibitory at high concentrations. However, they did suggest that frontalin was inhibitory at high concentrations and neutral at low concentrations. Borden et al. (1987) demonstrated that low release rates of frontalin or *exo*-brevicomin increased the response of *D. ponderosae* to *trans*-verbenol plus myrcene, but high release rates were inhibitory. They proposed that the key difference was in the response of different sexes to *exo*-brevicomin. Females rather than males were attracted by low release rates, and both sexes were inhibited at high release rates (Borden et al., 1987). Low release rates of frontalin were attractive to females, but high release rates were inhibitory to both sexes.

For both sibling species, *D. jeffreyi* and *D. ponderosae*, the male-produced compounds frontalin and *exo*-brevicomin seem to have a concentration-dependent message for both sexes. This suggests that there may be a general adaptive basis for the responses of these species, as well as other *Dendroctonus* species, to male-produced compounds for attraction at low concentrations. This may facilitate mating opportunities and location of available oviposition resources and deterence at high concentrations, which could reduce competition for limited resources. The results of the present study are suggestive of the role of the semiochemicals in communication by *D. jeffreyi*. However, the precision of that message can be finally determined only when the same enantiomers used by the beetles are incorporated into a field bioassay study.

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SUBTLETIES IN PERCEPTION AND DISCRIMINATION BY CABBAGE LOOPER MOTHS, *Trichoplusia ni*, OF THE TWO 14-CARBON ACETATE SEX PHEROMONE COMPONENTS

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Abstract—Three compounds that are emitted by virgin female cabbage looper moths, *Trichoplusia ni*, (Z)-7-dodecenyl acetate (Z7–12 : Ac), (Z)-7-tetradecenyl acetate (Z7–14 : Ac), and (Z)-9-tetradecenyl acetate (Z9–14 : Ac), are detected by antennal olfactory receptor neurons specialized to detect sex pheromone components. The principal evidence signifying that either or both of Z7–14 : Ac and Z9–14 : Ac have an effect on male behavior comes from an observed reduction in the numbers of males trapped by Z7–12 : Ac paired with a six-component mixture. The evidence conforms to a hypothesis that either or both of these compounds are the basis for discrimination between the two mixtures. However, scant evidence for this hypothesis was obtained in paired-source laboratory wind-tunnel assays, even at airborne concentrations of Z7–14 : Ac and Z9–14 : Ac that ranged up to 1000× that emitted by a female. Furthermore, there was little evidence from single-source laboratory or field trap assays confirming that Z7–14 : Ac and Z9–14 : Ac at or above natural stimulus strengths modify the moth's perception of Z7–12 : Ac. The data suggest that discrimination of small mixture differences may not be resolvable within the confines of a wind tunnel.

Key Words—*T. ni*, sex pheromone, behavior, perception, flight, orientation, trapping, wind tunnel.

INTRODUCTION

Usually, several compounds are found in the volatiles collected from virgin female moths (Mayer and McLaughlin, 1990). Typically, only a few of these

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clearly elicit or modify sexual behaviors. The cabbage looper moth, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) shows this well. Six compounds have been identified in the female's volatile emission, but one, (*Z*)-7-dodecenyl acetate (*Z*7–12 : Ac), is sufficient to elicit upwind flight and terminal sexual behaviors in wind tunnels and field traps (Linn et al., 1984, 1988; Landolt and Heath, 1987; Mayer and McLaughlin, 1991; Mayer et al., 1995). Three antennal olfactory neurons within long sensilla trichodea are specialized to detect *Z*7–12 : Ac, (*Z*)-7-tetradecenyl acetate (*Z*7–14 : Ac), and (*Z*)-9-tetradecenyl acetate (*Z*9–14 : Ac) (O'Connell et al., 1983; Grant and O'Connell, 1986; Mayer and Mankin, 1990; Mayer, 1993; O'Connell and Grant, unpublished). No other neurons within these long sensilla respond to female-emitted levels of (*Z*)-5-dodecenyl acetate (*Z*5–12 : Ac), dodecyl acetate (12 : Ac), and 11-dodecenyl acetate (11–12 : Ac), which also are found in the volatiles. On the other hand, others maintain that all six of the components are detected by neurons within the long sensilla trichodea (Todd et al., 1992), and there also appears to be a six-lobed macroglomerulus (Todd et al., 1995). The primary difference between these investigative group's findings, i.e., whether or not all six compounds are detected by three or six specialist neurons, centers about the stimulus strength and its relationship to the levels emitted by the female. Mayer (1993) shows that when airborne stimulus strengths exceed the levels that are emitted by the female, each of the three known specialists lose their natural selectivity and respond to all of the other pheromone components. The stimulus strengths deployed by Todd et al. (1995) for their studies significantly exceed the natural stimulus strength that can be found in the immediate downwind vicinity of a female. As a consequence of this intense stimulus strength, nonspecific responses can be elicited from the three already known specialists.

There are differences among the results of behavioral studies that are not easy to explain. For example, field trap assays conducted by Linn et al. (1988) show that mixtures of all six components resulted in more captures than traps baited with only *Z*7–12 : Ac. Mayer et al. (1995 and this report), however, find that *Z*7–12 : Ac alone captures at least the same number and perhaps more males than six-component and other mixtures. Furthermore, there are both qualitative and quantitative differences in elicited behaviors within wind tunnels (Linn et al., 1984; Landolt and Heath, 1987; Mayer and McLaughlin, 1991, 1993).

There also is much agreement among the studies cited above. All agree that *Z*7–12 : Ac, *Z*7–14 : Ac, and *Z*9–14 : Ac are detected by antennal specialist neurons. All agree that *Z*7–12 : Ac is plenipotent and required for sexual behavior. All would agree that *Z*7–14 : Ac and *Z*9–14 : Ac must have some function because natural selection probably would not conserve either the component or the specialist neuron for it if they did not. Previously, we were unable to demonstrate a role for *Z*7–14 : Ac and *Z*9–14 : Ac in cabbage looper sexual behavior (Mayer et al., 1995). The present paired and unpaired field-trap and wind-tunnel

assays were designed to uncover small effects of Z7–14 : Ac and/or Z9–14 : Ac on perception and discrimination of Z7–12 : Ac and its mixtures.

METHODS AND MATERIALS

Insects

Moths were reared on a semisynthetic medium (Guy et al., 1985). External sexually dimorphic characteristics were used to separate males and females during the pupal stage. After emergence, groups of 40–60 males were maintained in 25- × 25- × 25-cm Plexiglas and fiberglass screen cages until assayed. Two to four small plastic cups filled with a nutrient solution of 10% sucrose–water on cotton were provided for each cage. The cages were maintained in cabinets at 25–27°C, 70–80% relative humidity, and a 14L : 10-Dark photoperiod. Individual moths were assayed at 3–5 days of age, corresponding to the age of maximum male responsiveness (Shorey and Gaston, 1964). The assays began 4–7 hr into the scotophase (Shorey and Gaston, 1965). The flight tunnel, response standardization, behaviors, and experimental design were the same as described previously (Mayer and McLaughlin, 1991). The criterion of discrimination was a quantitative statistical difference in the numbers responding to two stimuli located side by side in the wind tunnel.

Wind Tunnel

Behavioral assays were conducted in a 183-cm long × 60-cm-ID cylindrical Plexiglas tunnel described in detail elsewhere (Mayer and Doolittle, 1995). The airspeed in the tunnel was maintained at 15–20 cm/sec. The plume produced from a dispenser loaded with tissue papers treated with NH_4OH and HCl is turbulent as defined by Mafra-Neto and Cardé (1995a,b).

Stimulus Control and Pheromone Mixtures

All compounds and mixtures for the laboratory behavioral assays were evaporated from calibrated glass dispensers (Mayer et al., 1987; Mayer, 1993). Two glass dispensers were connected to a glass Y-tube to provide accurate mixture concentrations (Mayer and McLaughlin, 1993). The Y-tube had a diverter port connected to a valved vacuum to start and stop the stimulus (Mayer and Doolittle, 1995).

Different airborne pheromone concentrations can result from mixtures of 12- and 14-carbon acetates because of their roughly 10-fold difference in evaporation rate. Thus, one dispenser was used to dispense the 12-carbon acetates and one for Z7–14 : Ac and Z9–14 : Ac. Both dispensers were purged with a constant flow of 200 ml/min air to eliminate or reduce potential concentration intensi-

fication within the dispenser during times when the stimulus was off. During periods when the stimulus was off, the stimulus-laden air was vented into the vacuum line. The stimulus was diverted into the center of the wind tunnel by closing off the vacuum.

A 10-cm-square piece of hardware cloth (0.635-cm mesh) was hung vertically from the nozzle of each blending device and a gray metal moth-shaped decoy was attached to the hardware cloth so that the posterior end of the simulated moth was above the nozzle (Shorey and Gaston, 1970).

Wind-Tunnel Assays

Discrimination. Two mixing devices with accompanying dispensers were placed in the center of the tunnel and released their contents parallel to the tunnel air flow 23 cm apart. No more than five males were flown on any day for each mixture pair, until a total of no less than 25 flights was accumulated. The criterion of discrimination is a significant deviation from equal response to the paired stimuli as assessed by chi-square analysis with Yates' correction (Steel and Torrie, 1960).

Perception. The responses by five males to a mixture were assayed in a wind tunnel on test days. The percentage response was analyzed with "days" as replications.

Behaviors

For behavioral assays, individual male moths were released from a holding cage centered on the stimulus source on the downwind hardware cloth screen. The behaviors observed were typical of moths flying upwind to a pheromone source and are characteristically a combination of opportunistic optomotor–chemoanemotaxis and/or a program of self-steered counterturning (Baker, 1990; Mafra-Neto and Cardé, 1995a,b). Behaviors scored were: plume orientation, close approach, source contact, and copulatory behaviors. In this report the behavioral stage labeled copulation occurs at the stimulus source and is characterized by ventrad abdominal thrusts, extrusion of claspers, and the eversion of both the lateral and terminal abdominal hairpencils. The tabulations of data are given for two behaviors only in this report because most of the males that oriented to the pheromone plumes continued upwind, contacted the source, and exhibited copulatory behaviors.

Field Traps Assays

Single traps were set 1.5 m high along roadways beside four cabbage fields near Bunnell, Florida, from April 23 to May 24, 1996, to establish whether or not the two 14-carbon acetates in the female emission affect perception of sex

pheromone by males (Mayer et al., 1995). The wind-oriented (WORT) traps, described by Mitchell et al. (1988) and Mayer et al. (1995), have two bucket traps suspended side by side 1.5 m high. The traps pivot in the wind to provide two side-by-side plumes, thus allowing moths flying upwind the opportunity to discriminate between baits. Bucket traps (International Pheromones Moth Traps, International Pheromone Systems, Merseyside, England) were used for both systems. Three Vaportape strips were placed in the bottom of each trap to kill the moths captured. The traps were serviced 11 times during the test period; there were six two-night intervals, three three-night, one four-night, and one five-night interval. The bait septum was changed weekly to minimize changes in pheromone composition and release rates.

The experimental layout for the single-trap assays was three rows of six traps in a large block of fields separated by roads and irrigation canals. Each row was separated by no less than 200 m and traps in each row were separated by 50 m. The row of four WORT traps was positioned between two of the single trap lines, but distant from either by at least 200 m. The position of the baits in each trap row was randomized at the beginning of the tests and then rotated systematically each time the traps were serviced. The WORT traps also were interchanged from left to right each time they were serviced.

Three bait mixtures were assayed. One mixture comprised all six putative sex pheromone components, one contained only the four 12-carbon acetates, and one bait was Z7–12 : Ac alone.

Septum Dosing

The sex pheromone mixtures were formulated with ratios based on Z7–12 : Ac that were the same as those determined by Bjostad et al. (1984) from airborne female moth emissions. The doses were pipetted into the well of unextracted rubber septa (Wheaton #224100-202, 11-mm sleeve stopper), and followed by 50 μl of hexane to ensure penetration of the pheromone (McDonough and Butler, 1983). The septa were attached to the lid of the bucket traps by a pin.

Pheromones

The pheromone components and their GLC analysis were provided through the courtesy of R. R. Heath, of this laboratory. The Z7–12 : Ac, 12 : Ac, and Z7–14 : Ac were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, and were 98.5%, 97.0, and 96.4% pure, respectively; Z9–14 : Ac was from Shin-Etsu Chemical Co., Tokyo, Japan, and was 89.8% pure; Δ11–12 : Ac was from Nu-Chek, Inc. P.O. Box 295, Elysian, Minnesota 56028, and was 99.1% pure; and the Z5–12 : Ac was synthesized and HPLC-purified to 99.5% purity by R. R. Heath.

Relationship of Stimuli to Female Emission Levels

For reasons discussed elsewhere (Mayer, 1993), it is necessary to reference the stimulus intensity to that of a female both to ensure the selectivity of the neuron that is specialized to detect the most important pheromone component, Z7–12 : Ac, the HS(a) neuron, as well as maintaining the relevance of the stimulus intensity to that of a female. Details for calculating and reproducing the female reference intensity for the wind tunnel and field trap assays can be found in Mayer (1993) and Mayer et al. (1995). All stimulus intensities are based on the airborne concentration of Z7–12 : Ac that may be found downwind from a female. Depending upon several variables, the airborne concentration of Z7–12 : Ac from a female is estimated to be approximately 1×10^{-11} M (Mayer, 1993). Pheromone dosages of Z7–12 : Ac and other 12-carbon pheromone acetates were prepared so that this airborne concentration was attained at the nozzle of the mixing device. Dosages of the 14-carbon acetates were varied depending upon the assay. Based on electroantennogram deflections, the average pheromone concentration may be diluted by a factor of approximately 8 about 2 m downwind from the dispenser (Mayer and McLaughlin, 1991).

Statistical Procedures

Paired Field-Trap WORT Assays. Trap counts were transformed to square roots to stabilize the variance. The figures and tables provide the actual counts, but the results of the analysis were based on the square-root transforms. Generally, there were only minor differences between the results from the transformed and untransformed data. The null hypothesis, that there was no difference in the response between the stimuli in WORT traps, and between Z7–12 : Ac in the two different WORT traps was tested by a paired t test.

Single Field-Trap Assays. Square-root transforms of the numbers of males captured per trap per night were analyzed by split-plot analysis of variance (ANOVA) with pheromone mixtures as main plot sources of variation and dates as subplot treatments (Steel and Torrie, 1960). Individual means were compared by Duncan's test and groups of means were compared by contrast statements within the analysis (SAS, PROC GLM) (Littell et al., 1991).

Paired Laboratory Wind-Tunnel Assays. The probability that responses between paired stimuli differ significantly from a 1 : 1 ratio was tested by chi-square with the Yates correction. The numbers of responses to paired stimuli in Tables 1 and 2 (below) are shaded where the null hypothesis was rejected at the $P = 0.05$ level.

Single-Source Wind-Tunnel Assays. For upwind responses to single stimulus sources in Table 3 (below), percentage responses and their square-root transforms were analyzed by SAS(PROC GLM and options) to assess differences among stimulus means (Littell et al., 1991). Subsets of the data with varying

TABLE 1. NUMBERS OF MALES APPROACHING AND COPULATING AT TWO SOURCES, ONE EMITTING SYNTHETIC FEMALE STIMULUS AND ANOTHER EMITTING FOUR 12-CARBON ACETATES WITH DIFFERING PROPORTIONS OF TWO 14-CARBON ACETATES NEAR THAT EMITTED BY FEMALES

Proportion of two 14-carbon acetates in test mixture[a]						
	Z9–14 : Ac					
Z7–14 : Ac	0	1	5	10	20	Totals[b]
Stage: Close approach						
0	**17 : 6**[c]	13 : 8	14 : 6	14 : 8	7 : 13	48 : 35
1	6 : 12	12 : 9	9 : 8	11 : 9	15 : 7	47 : 33
5	10 : 15	9 : 12	7 : 16	11 : 10	12 : 10	39 : 48
10	7 : 8	9 : 10	10 : 5	12 : 5	**18 : 6**	**49 : 26**
20	13 : 9	11 : 12	16 : 8	**18 : 7**	12 : 10	**57 : 37**
Totals[b]	36 : 44	41 : 43	42 : 37	**52 : 31**	**57 : 33**	**192 : 144**
Stage: Copulatory behavior						
0	12 : 5	12 : 9	12 : 5	12 : 6	3 : 10	39 : 30
1	3 : 9	10 : 8	4 : 9	9 : 8	8 : 6	31 : 31
5	8 : 11	8 : 11	**2 : 13**	6 : 10	7 : 10	**23 : 44**
10	6 : 7	5 : 8	7 : 5	12 : 5	12 : 6	36 : 24
20	13 : 8	8 : 11	13 : 4	14 : 9	12 : 9	47 : 33
Totals[b]	30 : 35	31 : 38	26 : 31	41 : 32	39 : 31	137 : 132

[a]The airborne concentration (M) at the dispenser nozzle and proportion of each component of the synthetic female-level mixture was: Z7–12 : Ac, 1.1×10^{-11}, 1.00; Z5–12 : Ac, 1.1×10^{-11}, 0.096; 11–12 : Ac, 3.1×10^{-12}, 0.028; 12 : Ac, 8.0×10^{-12}, 0.073; Z9–14 : Ac, 5.5×10^{-13}, 0.005; and Z7–14 : Ac, 1.2×10^{-12}, 0.011. The test mixture was the same except for the varying proportions of the two 14-carbon acetates.

[b]Totals in rows and columns do not include the zero levels.

[c]The first number within a cell is the number approaching, contacting, or copulating at the side emitting the synthetic female mixture; the second, separated from the first by a colon, is the number at the side emitting the test mixture. The total number of males assayed for each cell was 25. Boldface numbers deviate significantly from a 1 : 1 ratio.

amounts of Z7–14 : Ac and Z9–14 : Ac were examined further by regression analysis (SAS, PROC REG and options).

RESULTS

Field Assays

Paired WORT Traps. The differences in WORT trap captures that are illustrative of the subtle effect of Z7–14 : Ac and/or Z9–14 : Ac in discrimination of Z7–12 : Ac are found in comparisons III and IV of Figure 1. The differences in captures between the lures of these two pairings neared statistical significance,

TABLE 2. NUMBERS OF MALES APPROACHING AND COPULATING AT TWO SOURCES, ONE EMITTING SYNTHETIC FEMALE STIMULUS AND THE OTHER EMITTING FOUR 12-CARBON ACETATES AT DIFFERING BUT MUCH HIGHER PROPORTIONS OF TWO 14-CARBON ACETATES THAN EMITTED BY FEMALES

	Proportion of two 14-carbon acetates in test mixture[a]				
	Z9–14 : Ac				
Z9–14 : Ac	0	100	316	1000	Totals[b]
Stage: Close approach					
0	11 : 14[c]	14 : 10	15 : 7	12 : 12	41 : 29
100	16 : 8	14 : 7	14 : 8	8 : 10	36 : 25
316	11 : 14	15 : 8	11 : 11	8 : 11	34 : 30
1000	**4 : 18**	11 : 13	9 : 14	10 : 10	30 : 37
Total[b]	31 : 40	40 : 28	34 : 33	26 : 31	100 : 92
Stage: Copulatory behavior					
0	11 : 14	14 : 10	15 : 7	12 : 12	41 : 29
100	16 : 8	13 : 8	14 : 8	8 : 10	35 : 26
316	11 : 14	14 : 8	11 : 11	8 : 11	33 : 30
1000	**4 : 18**	9 : 14	9 : 14	10 : 10	28 : 38
Totals[b]	31 : 40	36 : 30	34 : 33	26 : 31	96 : 94

[a]The airborne concentrations and proportions of components are the same as for Table 1.
[b]Totals in rows and columns do not include the zero levels.
[c]The first number within a cell is the number approaching, contacting, or copulating at the side emitting the synthetic female mixture; the second, separated from the first by a colon, is the number emitting the test mixture. Boldface numbers deviate significantly from a 1 : 1 ratio.

but failed to exceed the requisite level. The result between these two pairings that is significant, however, is found in the difference in captures by Z7–12 : Ac. When paired with a six-component mixture, Z7–12 : Ac baited traps captured an average 2.1 males/night, but when paired with a four-component mixture, Z7–12 : Ac baited traps captured an average 10.5 males/night (Student's t-test, $N = 11$; $t = -2.297$; $P < 0.0367$).

WORT trap pairs I and II tested the hypothesis that males discriminate six-component mixtures that contain Z7–14 : Ac and Z9–14 : Ac and mixtures containing only the four 12-carbon acetates at either of two stimulus levels (Figure 1). The difference between the total number of males captured in either trap pair failed by a wide margin to exceed that necessary to reject the null hypothesis.

Unpaired Field Traps. Unpaired field traps measure the perceptual quality of pheromone mixtures (Figure 2). A total of 1025 males was captured over the duration of the assay. The main plot treatment variable of lure was highly significant ($P < 0.001$), as were the other main variables of fields and dates ($P < 0.001$). The two main interactions of the analysis, field and date and treatment and date, were not statistically different ($P = 0.231$ and 0.242, respectively).

TABLE 3. NUMBERS OF MALES APPROACHING AND COPULATING AT SOURCE EMITTING FOUR 12-CARBON ACETATES AT LEVELS EMITTED BY FEMALES BUT DIFFERING FEMALE EMISSION MULTIPLES OF TWO 14-CARBON ACETATES.

Female multiples of two 14-carbon acetates in test mixture[a]						
	Z9–14 : Ac					
Z7–14 : Ac	0	1	10	100	1000	Group Percentages[b]
Percentage close approach						
0	70 ± 15[c]	70 ± 11				70.0 ab
1	97 ± 3	68 ± 16	50 ± 21			83.5 a
10		52 ± 21	40 ± 21	30 ± 16		40.0 c
100			40 ± 15	16 ± 4	23 ± 13	27.8 c
1000				35 ± 17	53 ± 7	46.0 bc
Group percentages	86.2 a	63.8 b	51.8 bc	26.7 c	38.3 c	
Percentage copulatory behavior						
0	67 ± 16[c]	63 ± 11				65.0 ab
1	91 ± 4	68 ± 16				78.8 a
10		48 ± 19	40 ± 21	27 ± 17		37.5 cd
100			40 ± 15	16 ± 4	17 ± 13	25.6 d
1000				30 ± 17	47 ± 10	40.0 bc
Group percentages	80.0 a	60.0 a	37.7 b	31.7 b	24.0 b	

[a]The airborne concentrations and proportions of components are the same as for Table 1.
[b]Letters in the same column or row that have no letters in common are significantly different at $\alpha = 0.05$ by Ryan's test (Ryan, 1960).
[c]Mean ± standard error of the mean. The total number of males assayed for each cell ranged from 25 to 35.

Within this analysis, a contrast statement that compared the numbers captured with Z7–12 : Ac alone with the average of the four- and six-component mixtures confirmed the hypothesis that 1.0 mg Z7–12 : Ac alone captured significantly more males than the average of the two mixtures ($P < 0.024$). Two other contrast statements showed that: (1) there is not a statistically significant difference in the average numbers of males trapped between the six-component mixtures and the four-component mixtures at two levels ($P < 0.779$), and (2) there is a statistically significant difference in the average numbers of males trapped with the 1.0-mg and the 0.1-mg dosages ($P < 0.001$).

Laboratory Assays

Paired Sources. The first group of paired assays deployed the two 14-carbon acetates at proportions near those that are emitted by females (Table 1); the second group deployed them at unnaturally high stimulus strengths (Table 2). Generally, there are no trends toward discrimination at either near-natural or

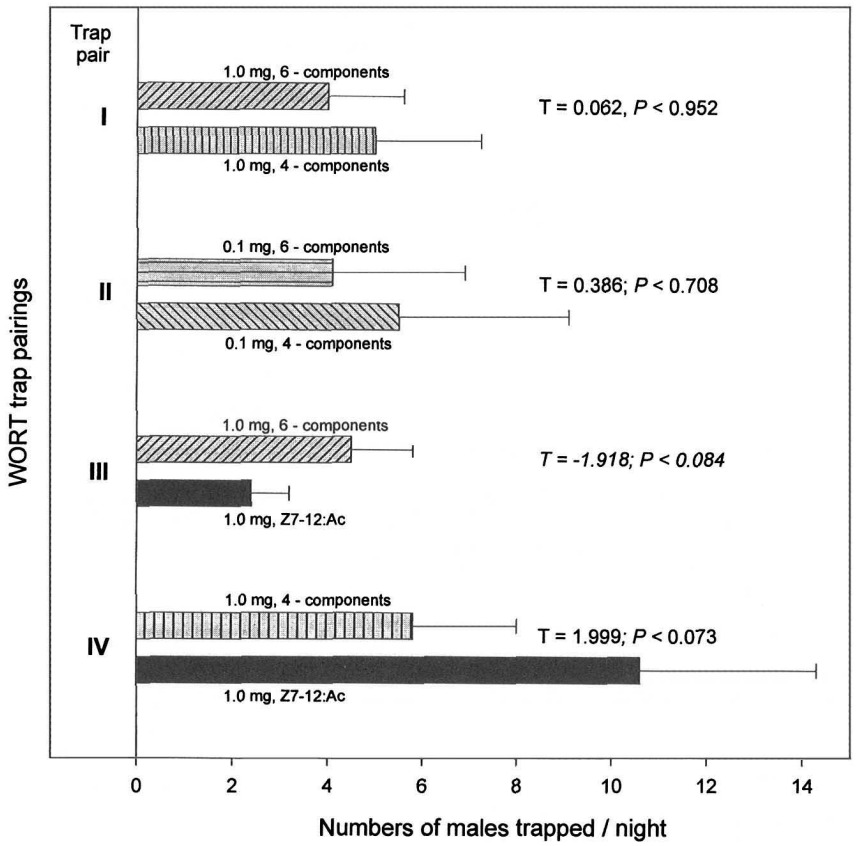

FIG. 1. Appraisal of pheromone mixture discrimination in field-trap assays by captures of male cabbage loopers in paired WORT traps baited with two levels of two mixtures and Z7–12 : Ac alone. Comparison I, discrimination of mixtures with and without the two 14-carbon acetates; II, discrimination of same mixtures as in pair I, but deployed at a lower dosage; III, discrimination of a six-component mixture and Z7–12 : Ac by itself; IV, discrimination of a four-component mixture and Z7–12 : Ac by itself. The lines above the bars are the standard error of the mean.

elevated concentrations of one or the other component, for example, within the matrices that might represent a central nervous system paradigm. Statistically, from tables of the binomial distribution, the probability of finding three or more significant pairs in 25 at the $\alpha = 0.05$ level of significance is 0.1271, and $P = 0.0341$ for four, etc. (Beyer, 1968). Thus, there is a 12.7% probability of spuriously declaring any three pairings to be different when they really may not be different.

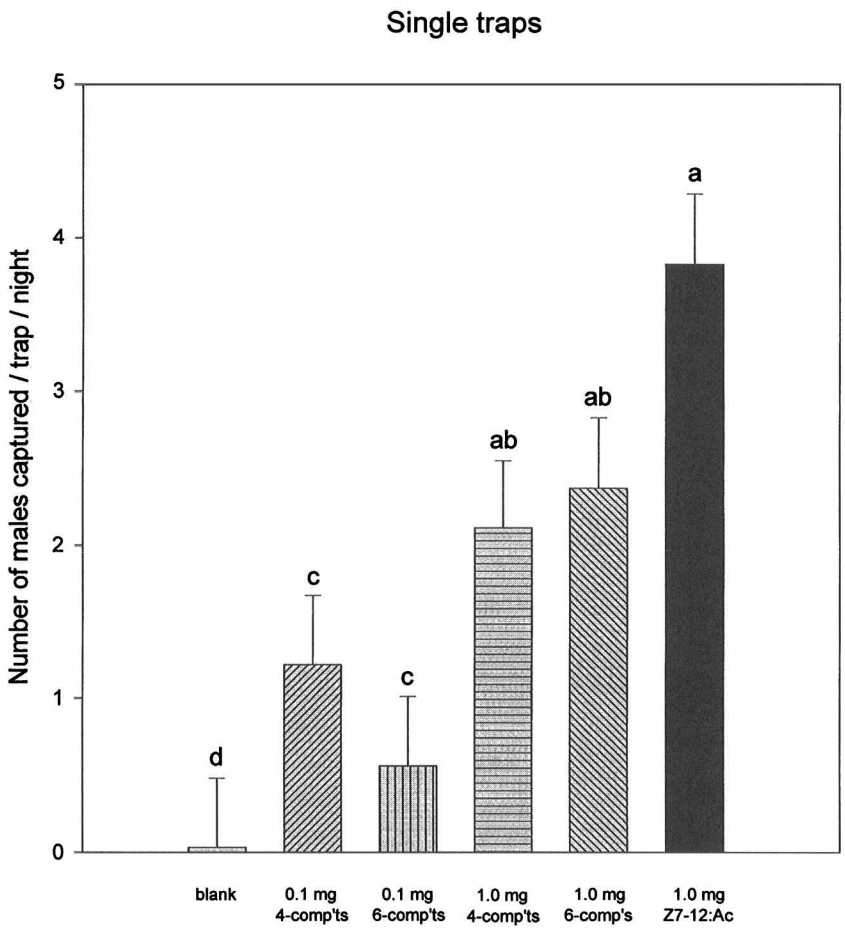

FIG. 2. Single-trap appraisal of the perceptible difference between innate sex pheromone recognition and two mixtures deployed at two dosage levels and Z7–12 : Ac by itself. Means sharing the same letter above the error bars are not significantly different at the $P = 0.05$ level by Duncan's test. The lines above the bars are the standard error of the mean.

Single-Source Quantities of 14-Carbon Acetates. Responses to unpaired stimulus sources are interpreted as measures of perception, in this case, the similarity of the aroma to that of the female. The reduction in male upwind flight responses at abnormally high female multiples of Z7–14 : Ac and/or Z9–14 : Ac tends to confirm the hypothesis that these two compounds affect perceptual differences (Table 3). Angular transforms of the data were analyzed by ANOVA

TABLE 4. PERCENTAGE RESPONSE AT FOUR CRITERION BEHAVIOR LEVELS TO RATIOS OF *Z*7–14 : Ac AND *Z*9–14 : Ac IRRESPECTIVE OF ASSAY CONCENTRATION

*Z*7–14 : Ac/*Z*9–14 : Ac Ratio	*N*	Response at criterion behavioral stage (%)[a]			
		Plume	Closing	Contact	Copulation
1 : 1	4	32.9 b	30.3 c	30.3 c	29.0 c
1 : 0	1	97.1 a	97.1 a	97.1 a	91.4 a
0 : 1	1	76.7 a	70.0 b	70.0 b	63.3 ab
1 : 10	3	45.9 b	42.4 b	42.4 bc	36.5 c
10 : 1	3	43.8 b	42.5 b	41.3 bc	40.0 bc

[a]Letters in the same column that have no letters in common are significantly different at $\alpha = 0.05$ by Ryan's test (Ryan, 1960).

and regression analysis and show that a second-degree model of *Z*7–14 : Ac and *Z*9–14 : Ac proportions fitted to the percentage of moths that copulated was highly significant ($P < 0.001$) but the value of $r^2 = 0.269$ was small. This makes the fitted equation practically useless for prediction of *Z*7–14 : Ac and *Z*9–14 : Ac levels.

Single-Source Ratios of 14-Carbon Acetates. Another way to analyze the data in Table 3 is to compare the behavior elicited by different ratios of *Z*7–14 : Ac and *Z*9–14 : Ac irrespective of their absolute amounts. There are five assays of a 1 : 1 ratio of *Z*7–14 : Ac : *Z*9–14 : Ac, one assay of a 1 : 0 ratio; one assay of a 0 : 1 ratio, three assays of a 1 : 10 ratio, and three assays of a 10 : 1 ratio (Table 4). The mean response elicited by 1 : 0 *Z*7–14 : Ac–*Z*9–14 : Ac was statistically greater ($P < 0.05$) than any other ratio.

DISCUSSION

The paired WORT field trap assays demonstrate the subtle difference in captures that manifests the cabbage looper's discrimination of *Z*7–12 : Ac and mixtures containing *Z*7–14 : Ac and *Z*9–14 : Ac. The discrimination of the six-component mixture and *Z*7–12 : Ac, manifest by fewer captures in traps baited with *Z*7–12 : Ac, was observed in our previous report but was disregarded until confirmed in these assays. This result is interesting because it was unaccompanied by any difference in captures by either of the two mixtures. There is little doubt that the two 14-carbon acetates are responsible for this result. What makes this result both subtle and perplexing is the lack of discrimination between six-component mixtures that have the 14-carbon acetates and four-component mixtures that lack the two 14-carbon acetates. The wind tunnel results indicate that *Z*7–14 : Ac and *Z*9–14 : Ac might affect mixture perception, the basis of the dis-

crimination of the six-component mixture and Z7–12 : Ac. In this respect, the data tend to agree with the redundancy notion of Linn et al. (1984; see their Figure 8).

Unpaired traps baited with Z7–12 : Ac alone captured at least as many, and averaged more, males than traps emitting any of the mixtures, a result agreeing with previous field trap data (Mayer et al., 1995) but differing with the data of Linn et al. (1988). It is hard to reconcile these two different results unless there are different genetic strains of cabbage loopers or significant differences between the emission of mixtures from their plastic caps and our rubber septa.

Extensive wind-tunnel experiments failed to provide clear evidence that the two sex pheromone components Z7–14 : Ac and Z9–14 : Ac account for the discrimination seen in the field between different mixtures of the 12-carbon acetates. This is true whether or not either of the two compounds were alone or mixed, or at female-emitted levels, or at multiples of more than 1000 times the emission of a female. This leads to an alternative conclusion that the proximity of the paired sources within the confined arena of the wind tunnel permits them to interact and reduce discrimination when the mixture differences are small. Nonetheless, discrimination of mixture differences can be observed when the differences between the stimuli are sufficiently large (Mayer and McLaughlin 1991, 1993; Valeur and Löfstedt, 1996).

There is discussion as to whether or not disruption of mating and communication on the field results from an interruption in one of three mechanisms: false trail following, adaptation or habituation, and/or an imbalance in sensory input (Minks and Cardé, 1988; Valeur and Löfstedt, 1996). Minks and Cardé argue that it is "... unwise to pursue the costly development of [disruptive] formulations that rely on less than the natural blend, unless field trials have verified disruptive efficacy...." We do not disagree with this statement, but from the standpoint of the cabbage looper, we ask, "what is the natural blend?" If the natural blend comprises compounds for which there are specialist neurons responding at stimulus strengths near that emitted by females, then there are only three components that comprise the natural blend of the cabbage looper. On the other hand, if it comprises those components that capture the greatest numbers in traps, then Z7–12 : Ac alone satisfies the criterion. On the basis of field trials, significant mating disruption was achieved with a mixture of just Z7–12 : Ac and (Z)-7-dodecenol (Z7–12 : OH) (Mitchell et al., 1997).

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ALKENES AS A SEXUAL PHEROMONE IN THE ALFALFA LEAF-CUTTER BEE *Megachile rotundata*

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Abstract—Because of its special biological features, the leaf-cutter bee *Megachile rotundata* is particularly well suited for breeding and use as a pollinator for fodder plants such as alfalfa. Its mating behavior has been extensively studied. Behavioral tests have shown that males explore any black spot as a potential mating partner. However, exclusive selection of young virgin females suggests involvement of olfactory cues in addition to visual stimuli. Experiments demonstrated that mating behavior could be induced in males by lures covered with pentane extracts of the epicuticular waxes of young females. These extracts contain 35% fatty acids, 26% alkanes, and 39% monoenes. Variations in the monoene subfraction allow distinction between young virgin females, which possess more 7-pentacosene and 9-pentacosene, and older females, which have more 5-monoenes. The findings show that male mate-seeking and copulatory activity is stimulated only by young female monoenes. A 40% increase in this activity was observed.

Key Words—Hymenoptera, Apoidea, *Megachile rotundata*, solitary bee, cuticular hydrocarbons, sexual behavior of males.

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INTRODUCTION

In a few species of bees, location of female mating partners by males is based only on visual stimuli (Butler, 1965; Meyer-Holzapfel, 1984), but, in general, olfactory cues are also involved. The existence of sex pheromones has been confirmed by a number of behavioral studies. Male *Macropis labiata* Panz. are attracted to a synthetic product simulating the odor of the female (Kullenberg, 1956). Male *Bombus pratorum* L. are able to locate females by odor without visual cues (Free, 1971). In several bee genera, virgin females have been shown to be more sexually attractive than older females, presumably because of sex pheromones (Kullenberg, 1973; Barrows, 1975; Alcock et al., 1976; Raw, 1976; Tengö, 1979; Cane and Tengö, 1981; Bergmark et al., 1984; Shimron and Hefetz, 1985; Smith and Ayasse, 1987; Tengö et al., 1988; Wcislo, 1992).

Few data have been provided concerning the chemical composition of sex pheromones in bees. 9-Oxodec-*trans*-2-enoic acid has been identified in *Apis mellifera* (Gary, 1962; Pain and Ruttner, 1963). In addition, certain aliphatic alcohols, esters, and terpenoid compounds have been linked to changes in sexual behavior and coupling in male *Andrena* and *Colletes* (Tengö, 1979; Cane and Tengö, 1981). In most cases, these compounds are found in both sexes, but sometimes they are found exclusively in females. Production of sex pheromone is generally attributed to the mandibular glands, as in *Xylocopa sulcatipes* Maa. (Velthuis and Gerling, 1980), but a macrocyclic lactone, a sex pheromone in *Lasioglossum zephyrum* (Smith et al., 1985), is produced by the Dufour gland. Contact pheromones have been identified in several insect species (Carlson et al., 1971; Finidori-Logli et al., 1996) but rarely in bees, the possible exception being male *Osmia rufa* L., which are attracted to females only at close proximity (Raw, 1976).

We are trying to identify sex pheromones in a megachilid bee, the leaf-cutter *Megachile rotundata* Fab. Because of its mode of nesting and efficiency in pollinating fodder plants, this species is bred and used for pollination of alfalfa (McGregor, 1976; Bohart, 1972). The biology (Stephen and Torchio, 1961; Klostermeyer 1982) and sexual behavior (Gerber, 1969) have been described, but nothing is known about sex pheromones. We performed experiments by using extracts obtained by soaking male and females in organic solvents. In addition to chemical analysis, we carried out behavioral tests to evaluate the attractivity of different female extract components to males.

METHODS AND MATERIALS

Insects. Pupae of a French variety of *M. rotunda* were supplied by the Zoological Laboratory of the Institut National de Recherche Agronomique in Luzignan, France. As previously described (Taséi and Carré, 1984), pupae were kept at 5°C and revived in an incubator at 30°C with relative humidity between 50%

and 80%. To maintain virginity, males and females were separated at emergence and kept in different wood and wire-mesh cages (35 × 35 × 40 cm) at a temperature of 30 ± 2°C, relative humidity of approximately 50%, and photoperiod of 14 hr light–10 hr dark. Animals were fed a sucrose solution.

Extraction. Extraction was performed on various-aged virgin females raised from pupae, on 3-day-old males raised from pupae, and on nesting females captured with pollen on the underside of the abdomen. Insects were soaked individually in 400 μl of pentane, dichloromethane, or methanol for 24 hr in a warm environment. Resulting epicuticular extracts were concentrated under a stream of nitrogen to obtain one bee equivalent per 50 μl of solution and stored at −18°C. Chemical composition was analyzed by GC from 2 μl of extract (1/200th of an insect). For behavioral tests, twenty virgin females and twenty 3-day-old males were soaked in 20 ml of pentane for 24 hr at room temperature.

Chemical Analysis. Gas chromatography was performed in a Delsi Nermag DN 200 system with a FID and a Chrompack capillary column (CPSil 5 WCOT, fused silica, 25 m × 0.22 mm). The carrier gas was helium, and oven temperature was programmed from 70 to 100°C at 30°C/min and from 100 to 320°C at 5°C/min. Maximum temperature was maintained for 10 min. Total program time was 55 min. Injector and detector temperatures were maintained at 280°C and 300°C, respectively. Injections were made in splitless mode (15 sec). The chromatograph was coupled to an Enica 31 integrator.

Gas chromatography–mass spectrometry (GC-MS) analysis was performed on a Hewlett-Packard 5890 GC coupled to a 5989A MS. The system was controlled by a HPUX MS Chemstation. Initial analysis was performed in the electron impact (EI) mode (70 eV) with the mass spectra scanned from m/z 100 to 600. GC was carried out by using an HP5 column (30 m × 0.25 mm). The carrier gas was helium at 2 bars. Injection was performed in the splitless mode (1 min). For chemical ionization (CI), methane pressure in the ionization chamber was maintained at 1.5 bars.

Alkenes were identified by retention time and relative position of equivalent chain length (ECL) in comparison with the *cis*-9-tricosene standard (Aldrich ref. 85,988-5). Double-bond positions were determined after alkylthiolation (DMDS) (Francis and Veland, 1981; Scribe et al., 1988). Identification of fatty acids was confirmed by base methanolysis (Bjostad et al., 1987) achieved by adding methanol and KOH (1%) in excess to the dried extract for 30 min, followed by 20 μl of pentane.

Mathematical Analysis. Relative peak proportions were calculated using Lotus 1,2,3 version 3.1 software from the areas given by the Enica 31 integrator coupled with the GC. Each peak was corrected prior to calculation using a flame ionization detector response coefficient as a function of the number of carbon atoms and the presence of a double bond (Bagnères et al., 1991). Principal component analysis (PCA) was carried out using 52 compounds found in extracts

as variables (Statgraphics Plus version 6 and Unistat Plus version 6 software). Variables from the different groups, i.e., 3-day-old males (N = 8), young virgin females (N = 16), old nesting females (N = 9), and one old virgin female were automatically normalized.

Preparation of Extracts. Three fractions were obtained from the young virgin female pentane extract by flash chromatography (Still et al., 1978). The solid (silica) phase of each fraction in Pasteur pipets (SDS, Chromagel 60A CC, 230 mesh) was placed in an aqueous solution of silver nitrate (20%) for 2 hr with either 2 ml of hexane (first fraction), 3 ml of a mixture of hexane–diethyl ether (90 : 10) (second fraction), or a mixture of 2 ml of a hexane–diethyl ether–acetic acid mixture (80 : 20 : 2) (third fraction). Fraction concentration was adjusted to one female equivalent (1 FE) per 50 μl of extract.

Behavioral Experiment I. Tests to assess sexual attraction were carried out in a Plexiglas olfactometer (OF) (10 × 15 × 100 cm). The OF consisted of a flight tunnel with a cage at each end and was divided into seven 10.5-cm sections numbered 0–6. In the cage at one end of the OF (section 0), five 2- to 7-day old, sexually active males were placed. In the cage at the other end (section 6), live virgin females or lures made with either the pentane, dichloromethane, or methanol extracts of virgin females or males were placed. A laminarlike airflow (0.15 m/sec) was created in the tunnel by a fan blowing air through horizontal bars behind the cage containing the stimulus. The lure was a 2- × 2-cm piece of filter paper treated with pheromone extract and suspended from the door in the ceiling of the cage. All tests were carried out between 10 AM and 3 PM since peak bee activity has been observed during this period (Szabo and Smith, 1972). Since testing was performed indoors, two 23-W neon light strips and one 75-W incandescent bulb were placed 25 cm above the OF to enhance light intensity. Males were always allowed 15 min to adapt to the new environment. Controls were made with pure air for virgin female tests and with corresponding solvents for the lure tests. In all cases, the solvent containing one equivalent insect was left to evaporate outside the OF. Numerous preliminary trials (see Results) to determine optimal test conditions showed that emerged females always excited several-day-old males. The initial position of the males was noted, and displacement was measured by counting the number of insects per section every 30 sec for 5 min.

From the resulting data, an index of relative attraction (IRA) was calculated by the following equation (from Zagatti, 1985):

$$\mathrm{IRA} = 100 \times \frac{\sum_{i=0}^{7} in_{it} - \sum_{i=0}^{7} in_{i0}}{\sum_{i=0}^{7} (7 - i)n_{i0}}$$

where n is the number of males, i is the section in the OF (from 0 to 7), and t is the time, so n_{it} is the number of males in the section at time t. Index values were compared using the Mann-Whitney nonparametric test (Schwartz, 1988). The null hypothesis was that there is no difference between index values obtained in tests using stimuli (females or lures) and corresponding controls (air or solvent only). The alternative hypothesis was that higher index values are obtained in tests using stimuli than in respective control tests and that this is evidence of sexual attraction.

Behavioral Experiment II. Male mating behavior was assessed in a rectangular Plexiglas box (11 × 13.5 × 15 cm) closed at each end by a fine wire screen. Light and temperature conditions were the same as in experiment I. In each test, five males were placed in the cage and allowed 15 min to adapt to the new environment. Next, one live 1- to 2-day-old female or one lure was placed in the cage. The lures used were treated with whole pentane extracts obtained from females and males of the same age and three fractions obtained from the female extracts. One equivalent insect per 50 μl of extract was used. Controls were performed using no stimulus instead of a live female, the same amount of pure pentane instead of the whole extracts, and a hexane–diethyl ether solution (80 : 20) instead of the fractions. Between eight and ten 5-min tests and controls were carried out for each stimulus. Animal behavior was filmed using a Sony video camera (model DCX-1820 P) connected to a VHS video recorder (model NV-FS 100 HQ). Films were played back and studied on a Sony monitor (model NM 2010 QM). The number of take-offs, i.e., times that males either leapt into the air or flew, was considered as indicative of increased activity.

Statistical analysis consisted of classifying the males into groups according to number of take-offs. Each stimulus was then combined with its respective control, and the median, dividing males into two groups with the same number of take-offs on each side, was determined. The number of take-offs in each test and control experiment was then counted separately on either side of the median. Resulting counts were compared using the χ^2 test (Siegel, 1956).

Behavioral Experiment III. Preliminary field tests were performed in a 50-cm^3 cage on a sunny terrace. Six males were placed in the cage, and the stimulus was placed in an open Petri dish on a 30-cm-high pedestal in the center of the cage. Tests were carried out by using pieces of filter paper or a washed dead female soaked in 50 μl of the different fractions of young virgin female extract. Controls were performed with 50 μl of pure solvent (Kullenberg, 1973). Male activity within a 10-cm radius around the odor source was observed over a 5-min period. Three patterns of behavior were distinguished: behavior I—flying close to the stimulus (10 cm) without changing direction; behavior II—changing direction when passing the stimulus; and behavior III—hovering in front or circling around the stimulus.

Results were compared using the χ^2 test.

RESULTS

Chemical Analysis. The quantity of each compound found in cuticular extracts from young females, older females, and males is listed in Table 1 and represented by a typical GC trace in Figure 1. In agreement with previous findings in insects (Blomquist et al., 1987; Lockey, 1988), the main cuticular components in *M. rotundata* are linear alkanes (16–26% of the total lipids found in the extracts), monomethylalkanes (0.5%), alkenes (24–37%), fatty acids (20–38%), and trace amounts of aliphatic esters. Most *n*-alkanes contain between 21 and 31 carbon atoms plus tritriacontanes. Other *n*-alkanes are present only in trace amounts. Each of the more abundant *n*-alkanes, from *n*-C_{23} to *n*-C_{33}, is preceded by a series of monoene isomers with differing double-bond positions at carbon 5, 7, 9, 11, or 13. Some double bonds were identified only by retention time, their amounts being too small for the DMDS derivatization.

Acids with saturated, monounsaturated, or diunsaturated hydrocarbon linear chains were identified. They are lauric acid (peak 7 in Table 1), tetradecenoic acid (peak 13), myristic acid (peak 14), palmitoleic acid (peak 17), palmitic acid (peak 18), linoleic acid (peak 21), oleic acid (peak 22), and stearic acid (peak 24). The position of unsaturation of tetradecenoic acid could not be determined. These findings were confirmed by base methanolysis after derivatization of total extracts, showing the presence of methyl esters of lauric, myristic, palmitoleic, palmitic, linoleic, oleic, and stearic acids.

Trace amounts of aliphatic esters were found only in males and young females. They correspond to ethyl esters with mass spectra characterized by an m/z 88 ion due to a McLafferty rearrangement. Comparison with commercial standards identified these products as ethyl laurate, myristate, palmitate, oleate, and stearate (compounds 8, 15, 19, 25, and 26). Four long-chain alcohol esters were observed only in females: octyl and decyl oleates (compounds 61 and 76), octyl stearate (64). Identification as octyl and decyl oleates and octyl stearate was confirmed by comparison with spectra obtained with synthetic products (Attygalle et al., 1986).

Four undetermined compounds (peaks 11, 13, 23, 33) with mass spectra characterized by an m/z 99 base peak were observed mostly in males with the exception of the most abundant compound (23), which was occasionally found in young females.

Multivariate Analysis. Principal component analysis (Figure 2) revealed three distinct clusters of bees. The main discriminating factor was the relative proportion of *n*-alkanes and monoenes. Fatty acids were not useful. Correlation between products of the epicuticular wax and the two main components of the multivariate analysis are shown in Table 2. The first principal component (axis 1 in Figure 2) accounted for 28.2% of the total variation and separated females into two groups, i.e., young virgin females (positive part of axis) versus the 15-

TABLE 1. DIAGNOSTIC IONS, DETERMINATION AND QUANTIFICATION OF COMPOUNDS EXTRACTED FROM EPICUTICLE OF *Megachile rotundata* MALES AND FEMALES[a]

Peak	ECL	Lipid	Young females		Old females		Males		Diagnostic ions MS/EI	CI
			%	± %	%	± %	%	± %		
1	11.95	1-Dodecene	tr						*61,168,201,262*	167
2	12.00	*n*-Dodecane	tr						170	169
3	13.00	*n*-Tridecane	tr						184	183
4	13.95	1-Tetradecene	tr						*61,196,229,290*	195
5	14.00	*n*-Tetradecane	tr				tr		198	197
6	15.00	*n*-Pentadecane	tr				tr		212	211
7		Lauric acid	tr						60,73,129,157,171	200
8		Ethyl Laurate	tr				tr		88,101,115,157,183,185	228
9	16.00	*n*-Hexadecane	tr				tr		226	225
10	17.00	*n*-Heptadecane	tr						240	239
11		Unknown					tr		99	
12		Unknown					tr		99	
13		9-Tetradecenoic acid	tr						55,60,69	226
14		Myristic acid	0.03	0.04	0.14	0.07	0.37	0.34	60,73,129,185,228	228
15		Ethyl Myristate	tr				tr		88,101,115,157,211,213,256	256
16	19.00	*n*-Nonadecane	tr						268	267
17		9-Hexadecenoic acid	1.73	0.98	1.3	0.38	1.02	1.24	55,60,73,83,129,194,236,(254)	254
18		Palmitic acid	3.45	0.86	1.9	0.22	2.73	1.83	55,60,73,125,157,171,185,213,256	256
19		Ethyl Palmitate	tr				tr		88,101,115,157,239,241,284	284
20	21.00	*n*-Heneicosane	0.31	0.14	0.53	0.3	0.04	0.03	296	295
21		Linoleic acid	13.32	4.3	21.54	4.25	7.24	4.49	55,60,67,79,82,95,108,121,149,222,280	280
22		Oleic acid	14.09	1.2	12.02	1.64	7.45	3.14	55,60,69,83,97,112,125,151,222,264,282	282
23		Unknown	0.55	0.3	0.7	0.15	38.7	21.22	99,110,111,145,155,170,227,271	
24		Stearic acid	2.39	0.35	1.47	0.24	0.83	0.71	55,60,69,84,97,112,129,152,185,222,284	284
25		Ethyl Oleate	tr		tr		tr		55,69,83,88,97,101,111,264,265,310	310
26		Ethyl Stearate	tr		tr		tr		88,101,115,157,267,269,312	312
27	22.00	*n*-Docosane	0.06	0.02	0.07	0.06	0.04	0.03	310	309

TABLE 1. CONTINUED

Peak	ECL	Lipid	Young females %	Young females ± %	Old females %	Old females ± %	Males %	Males ± %	Diagnostic ions MS/EI	CI
28	22.69	9-Tricosene	0.22	0.06	0.07	0.04	0.05	0.05	*173,243,322,416*	321
29		Unknown			0.14	0.04				
30	22.76	7-Tricosene	0.63	0.1	0.14	0.07	0.17	0.08	*145,271,322,416*	321
31	22.87	(5)-Tricosene	0.15	0.04	0.41	0.12	0.07	0.04	322	321
32	23.00	*n*-Tricosane	3.49	0.5	1.63	0.33	0.89	0.42	324	324
33		Unknown					0.20	0.10	99	
34		Unknown			0.07	0.06				
35	23.68	9-Tetracosene	0.13	0.03	0.24	0.06	0.08	0.03	*173,257,336,430*	335
36	23.76	7-Tetracosene	0.16	0.02	0.02	0.03	0.09	0.04	*145,285,336,430*	335
37	23.86	(5)-Tetracosene	tr		0.72	1.66	0.06	0.03	336	335
38		Unknown			0.19	0.11	0.02	0.03		
39	24.00	*n*-Tetracosane	0.42	0.04	0.35	0.06	0.24	0.08	338	337
40	24.62	(11)-Pentacosene	0.52	0.3			0.22	0.13	350	349
41	24.67	9-Pentacosene	7.93	1.07	5.35	0.97	3.08	0.94	*173,271,350,444*	349
42	24.76	7-Pentacosene	11.61	1.4	6.87	1.02	5.11	1.86	*145,299,350,444*	349
43	24.87	5-Pentacosene	3.27	0.6	7.82	1.06	2.42	1.12	*117,327,350,444*	349
44	25.00	*n*-Pentacosane	11.61	1.54	9.41	3.47	6.49	2.02	352	351
45	25.33	11- + 13-Methylpentacosane	0.17	0.02	0.27	0.06	0.1	0.05	168,196,224,351(M-15)	365
46	25.52	13-Hexacosene	0.03	0.03	0.2	0.08			*229,364,458*	363
47	25.60	(11)-Hexacosene	0.19	0.04	0.22	0.07	0.1	0.03	364	363
48	25.68	9-Hexacosene	0.19	0.03	0.19	0.03	0.12	0.03	*173,285,364,458*	363
49	25.77	(7)-Hexacosene	0.27	0.03	0.05	0.06	0.26	0.11	364	363
50	25.88	(5)-Hexacosene	0.1	0.02	0.34	0.04	0.15	0.05	364	363
51	26.00	*n*-Hexacosane	0.41	0.11	0.34	0.08	0.26	0.09	366	365
52		Unknown	0.22	0.07	0.61	0.25	0.09	0.05		
53		Unknown	0.23	0.07	0.08	0.11	0.05	0.07		
54	26.52	13-Heptacosene	0.32	0.06	0.56	0.25	0.26	0.22	*229,243,378,472*	377
55	26.60	11-Heptacosene	2.74	0.28	5.2	1.16	1.95	0.72	*201,271,378,472*	377
56	26.69	9-Heptacosene	1.55	0.27	2.04	0.29	1.09	0.44	*173,299,378,472*	377

57	26.77	7-Heptacosene	3.96	0.47			3.9	1.97	*145,327,378,472*	377
58	26.88	5-Heptacosene	2.05	0.27	4.72	0.55	2.59	0.87	*117,355,378,472*	377
59	27.00	*n*-Heptacosane	5.53	0.58	5.25	1.35	3.67	1.31	380	379
60	27.34	11- + 13-Methylheptacosane	0.22	0.09	0.11	0.01	0.07	0.04	168,196,224,252,379(M-15)	421
61		Octyl Oleate	tr		tr				180,222,264,312,351,394	395
62		Unknown	tr		tr					389
63	27.67	Octacosene					0.06	0.03	392	391
64		Octyl Stearate	tr		tr				112,128,185,241,285,396	397
65		Octacosene					0.05	0.01	392	391
66	27.93	Octacosene					0.08	0.03	392	391
67	28.00	*n*-Octacosane	0.14	0.08	0.16	0.12	0.55	0.04	394	393
68	28.46	Nonacosene	tr		tr				406	405
69	28.56	13-Nonacosene	tr		0.1	0.05	0.04	0.03	*229,271,406,500*	405
70	28.61	11-Nonacosene	0.54	0.03	0.75	0.21	0.73	0.37	*201,299,406,500*	405
71	28.67	(9)-Nonacosene	0.04	0.05	0.35	0.11	0.14	0.08	406	405
72	28.79	7-Nonacosene	0.04	0.11	0.04	0.04	0.84	0.46	*145,355,406,500*	405
73	28.91	(5)-Nonacosene	0.28	0.07	0.55	0.09	0.52	0.15	406	405
74	29.00	*n*-Nonacosane	2.52	0.42	2.54	0.66	2.34	0.97	408	407
75	29.34	13- + 15-Methylnonacosane	0.06	0.04	0.13	0.06	0.06	0.03	196,224,252,407(M-15)	421
76		Decyl Oleate	tr		tr				264,292,311,422	423
77		Unknown	tr		tr					
78	30.00	*n*-Triacontane	tr		0.1	0.02	0.11	0.05	422	421
79	30.61	(11)-Hentriacontene	tr		0.03	0.04	0.09	0.05	434	433
80	30.68	(9)-Hentriacontene	tr		0.09	0.04	0.06	0.06	434	433
81	30.80	7-Hentriacontene	tr		0.07	0.04	0.25	0.24	*145,383,434,528*	433
82	30.90	(5)-Hentriacontene	tr		0.08	0.04	0.07	0.05	434	433
83	31.00	*n*-Hentriacontane	0.98	0.38	1.04	0.26	1.04	0.59	436	435
84	31.34	13- + 15-Methylhentriacontane	tr		tr				196,224,252,280,435(M-15)	449
85	32.00	*n*-Docotriacontane	tr						450	449
86	32.79	(7)-Tritriacontene	tr						462	461
87	32.90	(5)-Tritriacontene	tr		tr				462	461
88	33.00	*n*-Tritriacontane	0.7	0.31	0.72	0.3	0.69	0.33	464	463
89	35.00	*n*-Pentatriacontane	tr						492	491

[a]The series of diagnostic ions in italics are taken from mass spectra obtained by DMDS; double bond positions in parentheses were not confirmed. ECL = equivalent chain length; MS/EI = mass spectrum/electron impact, CI = chemical ionization. % = relative proportions, ± % = standard deviation.

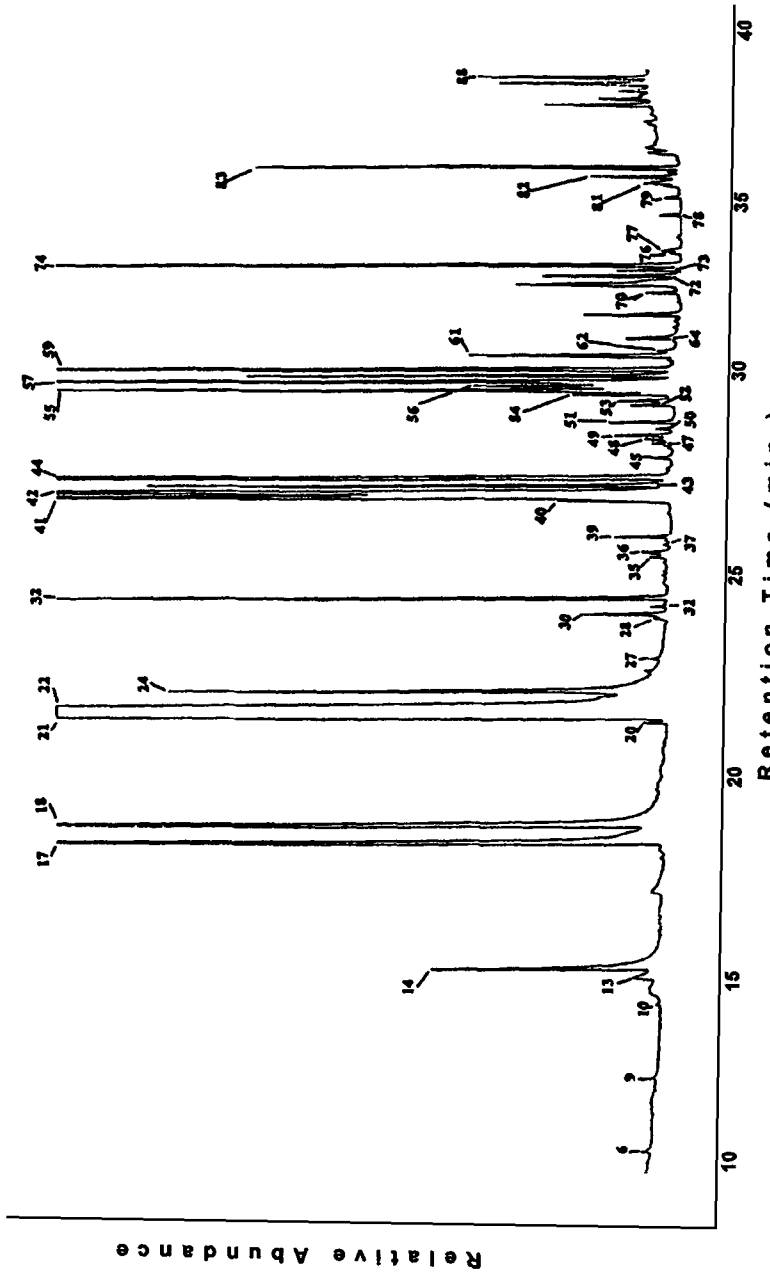

FIG. 1. GC trace of one young female *Megachile rotundata* from a total extract. Peak numbers refer to Table 1.

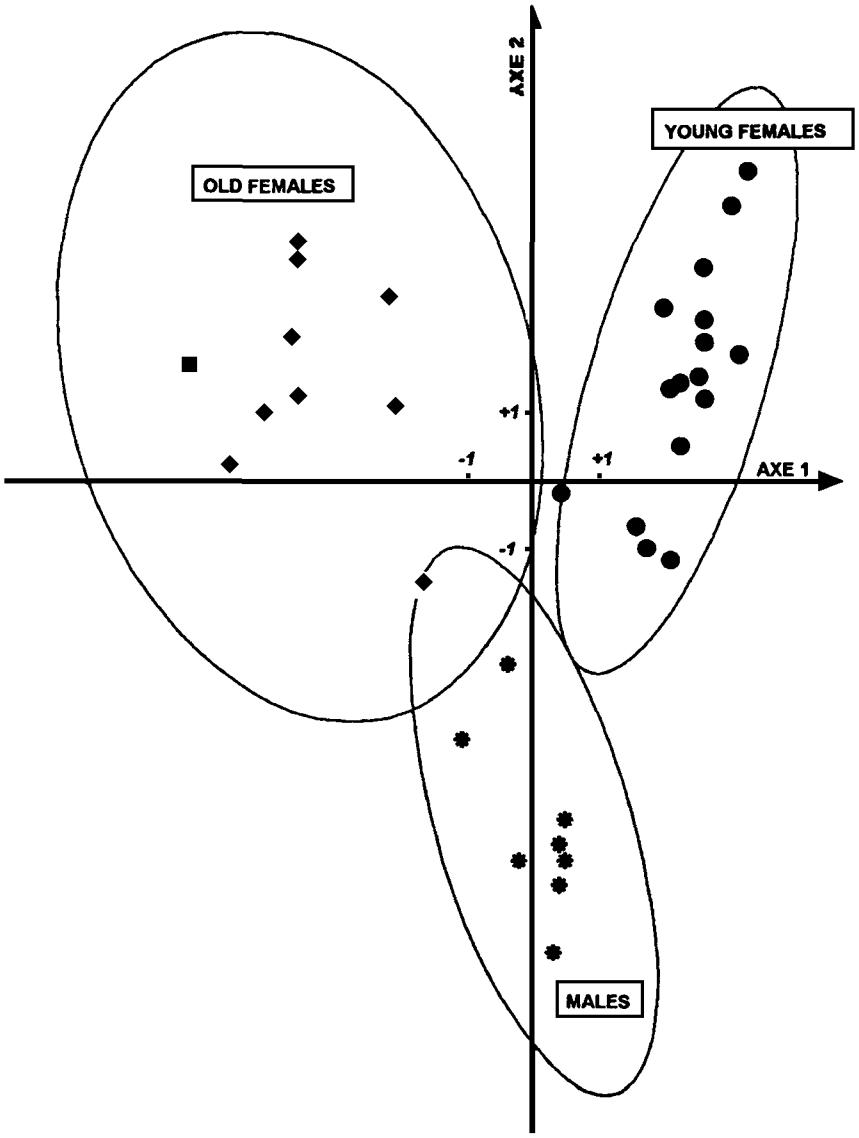

FIG. 2. Multivariate analysis of cuticular hydrocarbons and fatty acids (52 variables normalized) of 26 female and 8 male *Megachile rotundata*. Projection in two principal components of the different categories of bees (●: young females, ♦: older virgin females, and ■: older nesting females, ✻: males). The tolerance ellipses contain 95% of observations.

TABLE 2. SIMPLE CORRELATIONS BETWEEN CUTICULAR LIPIDS AND THE TWO FIRST PRINCIPAL COMPONENTS OF MULTIVARIATE ANALYSIS

Lipid	Component 1	Component 2
Hexadecenoic acid	0.082	−0.106
Palmitic acid	0.333	−0.119
n-Heneicosane	−0.337	0.480
Linoleic acid	−0.483	0.152
Oleic acid	0.225	0.627
Stearic acid	0.422	0.538
9-Tricosene	0.564	0.494
Unknown	−0.824	0.360
7-Tricosene	0.776	0.531
5-Tricosene	−0.709	0.493
n-Tricosane	0.622	0.679
9-Tetracosene	−0.584	0.562
7-Tetracosene	0.820	0.111
5-Tetracosene	−0.343	0.198
Unknown	−0.718	0.292
n-Tetracosane	0.246	0.775
11-Pentacosene	0.527	0.178
9-Pentacosene	0.450	0.761
7-Pentacosene	0.584	0.670
5-Pentacosene	−0.801	0.511
n-Pentacosane	0.329	0.763
11- + 13-Methylpentacosane	−0.398	0.624
13-Hexacosene	−0.695	0.285
11-Hexacosene	−0.141	0.438
9-Hexacosene	0.004	0.685
7-Hexacosene	0.715	0.196
5-Hexacosene	−0.935	0.206
n-Hexacosane	0.243	0.511
Unknown	−0.667	0.388
Unknown	0.414	0.168
13-Heptacosene	−0.564	0.181
11-Heptacosene	−0.579	0.399
9-Heptacosene	−0.333	0.542
7-Heptacosene	0.760	−0.193
5-Heptacosene	−0.875	0.278
n-Heptacosane	0.129	0.642
11- + 13-Methylheptacosane	0.275	0.206
n-Octacosane	−0.078	−0.333
13-Nonacosene	−0.798	0.043
11-Nonacosene	−0.299	−0.208
9-Nonacosene	−0.754	0.070
7-Nonacosene	0.421	−0.516
5-Nonacosene	−0.674	−0.266

TABLE 2. CONTINUED

Lipid	Component 1	Component 2
n-Nonacosane	0.023	0.128
13- + 11-Methylnonacosane	−0.422	0.041
n-Triacontane	−0.635	−0.382
11-Hentriacontene	−0.250	−0.597
9-Hentriacontene	−0.604	−0.256
7-Hentriacontene	−0.130	−0.495
5-Hentriacontene	−0.566	−0.367
n-Hentriacontane	−0.049	−0.174
n-Tritriacontane	0.022	−0.165

day-old virgin female and the nesting females (negative part of axis). A progressive age-related change in the chromatographic profile of cuticular hydrocarbons was observed in females. This evolution, which was independent of the activity (nesting or not) involved a decrease in the relative proportion of 7-monoenes (7-tetracosene, 7-tricosene, 7-heptacosene, and 7-hexacosene) in favor of the 5-monoenes (5-hexacosene, 5-heptacosene, and 5-pentacosene). The proportion of 7-monoenes fell from 7% of total hydrocarbons in the young virgin females to less than 5% in older females. This decrease was offset by an increase of the 5-monoenes: 7% for young females versus 21% for older females, so that the relative proportion of the monoenes remained unchanged.

The second principal component (axis 2 in Figure 2) accounted for 18.3% of the total variation and separated males (negative part of axis) and females (positive part of axis). The relative proportion of heavy hydrocarbons (more than 28 carbon atoms) was higher in males, while the relative proportion of certain light *n*-alkanes (*n*-tricosane, *n*-tetracosane, and *n*-pentacosane) was higher in females. The latter *n*-alkanes along with 9- and 7-pentacosenes were most correlated with the positive part of axis 2. The relative proportion of 9- and 7-pentacosenes was higher in young females (20%) than in older females and males (12%). As shown in Figure 2, the homogeneity of these three groups was confirmed by confidence ellipses calculated with a risk level of 5%.

Preliminary Trials. When females were placed in cages containing previously passive males, rapid changes in male behavior were observed, including antenna movement, increased crawling speed, increased flying activity, and unusual flight patterns. Males were attracted to and explored any dark area, supposedly in search of the female. Similar mate-seeking behavior was observed by using pentane extracts from young virgin females but not by using methanol and CH_2Cl_2 extracts. After locating a female, the male attached itself to the partner's back and the couple fell to the floor. Unreceptive females often were able to escape by pushing the male away by using their back legs. When observed,

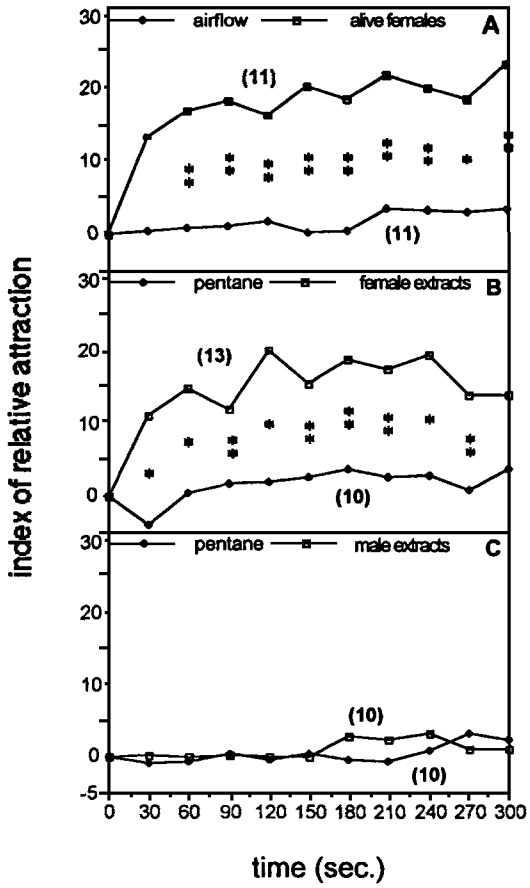

FIG. 3. Index of relative attraction of five *Megachile rotundata* males for live young females (A), pentane extracts of young females (B), and male extracts (C) (one equivalent insect). The controls (♦) correspond to equivalent quantity deposits (50 μl) of the pure solvent extract (pentane for B and C) or to the flow of pure air for the tests carried out with alive females (A). The curves are compared using the Mann-Whitney nonparametric test; significant difference: $^{*}P < 0.05$ or $^{**}P < 0.01$. Number of tests is in parentheses.

copulation lasted only about 12 sec. Afterwards, males and females remained attached with no further attempt to mate for up to 14 min. Males that failed to locate a female partner attempted to mate with each other, sometimes forming long lines on the floor of the OF.

Behavioral Experiment I. Curves obtained from index values are presented in Figure 3. The only curves that were significantly different from controls were

those from live females (A) and pentane extracts of young virgin females (B). The controls were pure air (A) and pure pentane (B). Curves from tests with pentane extracts of males (C) and methanol and dichloromethane extracts of females (not shown) were not significantly different from controls. These findings confirm that live females and pentane extracts of young virgin females are sexually attractive to males. The null hypothesis was rejected with a risk of error of less than 0.01 ($P < 0.01$) for live females and less than 0.05 ($P < 0.05$) for pentane extracts from young virgin females.

Since females and lures were confined to one end of the OF, contact and copulation were not possible. Under these conditions, males landed on the screen in front of the females or lure. They frequently made one or more end-to-end trips in the OF and inspected any dark spot, including other males, to determine if it was a female. In many cases, males chased each other in flight and could be observed in couples on the floor of the OF. This behavior was not observed in tests of extracts from males.

Behavioral Experiment II. The number of take-offs counted in each test and control experiment are given in Table 3, along with corresponding χ^2 values. The null hypothesis was rejected by comparison of test and control data obtained

TABLE 3. DETERMINATION OF MALE *Megachile rotundata* ACTIVITY BY χ^2 COMPARISON OF NUMBER OF TAKE-OFFS DURING 5-MIN TESTS USING DIFFERENT STIMULI AND CONTROLS

Control and stimulus	Take-offs by less active males (Left side of the median)	Take-offs by highly active males (Right side of the median)	χ^2	Risk (r)
Without stimulation	105	54	25.12	<0.001[a]
Live females	84	130		
Pentane	76	32	22.71	<0.001[a]
Whole female extract	69	102		
Pentane	61	47	2.81	>0.05
Whole male extract	42	54		
Hexane–ether	90	78	2.3	>0.01
Female alkanes	61	77		
Hexane–ether	126	42	70.41	<0.001[a]
Female alkenes	66	146		
Hexane–ether	84	84	1.02	>0.1
Female fatty acids	63	75		

[a]Significant difference.

TABLE 4. DETERMINATION OF MALE *Megachile rotundata* ACTIVITY BY χ^2 COMPARISON OF NUMBER OF TIMES EACH BEHAVIOR PATTERN WAS OBSERVED IN TESTS USING DIFFERENT ODORANT STIMULI AND IN CONTROL PURE SOLVENT (80 : 20 HEXANE–ETHER)

Stimulus	Behavior types			Approaches (*N*)	χ^2
	I	II	III		
Filter paper	0	9	0	9	
Pure solvent	13	11	1	25	
Female extract	8	18	12	38	9.93[a]
Female alkanes	6	10	0	16	0.51
Female alkenes	13	7	17	37	13.29[a]
Female fatty acids	8	4	2	14	0.45

[a]Significant difference with $P < 0.01$.

from live females, pentane extracts of females, and the alkenes of the female extracts. Under these conditions, the χ^2 increment was about 25 ($P < 0.001$). The null hypothesis was not rejected by comparison of test and control data obtained from male extracts and alkane and acid fractions from female extracts. Under these conditions, the χ^2 increment was less than 3.6 ($P > 0.05$). These findings confirm that live young virgin females and pentane extracts and alkenes from live young virgin females increase activity in sexually active males, as judged by the number of take-offs.

Behavioral Experiment III. The number of times each of the behavioral patterns was observed is summarized in Table 4. Since some values obtained in the χ^2 test were less than 5, application of the Yates correction coefficient was required (Schwartz, 1988). The null hypothesis, i.e., stimulus does not affect male behavior, was rejected in tests using young virgin female extracts ($P < 0.02$) and alkenes from young virgin female extracts ($P < 0.01$). Overall occurrence of the three behavior patterns was highest in tests of young virgin female extracts (38) and alkenes from young virgin female extracts (37). Values were intermediate in tests of pure solvent (25), and low in tests of alkane (16) and acid (14) fractions. Hovering and circling (behavior III) confirm the interest of the male in the stimulus contained in the Petri dish.

DISCUSSION

All three behavioral experiments demonstrate that male mating behavior is elicited by the presence of live females, whole pentane extracts from live females, and the alkene fraction of pentane extracts from live females. Based on these observations, we conclude that females emit a sex pheromone that acts on males.

Experiment I (OF tests) was designed to evaluate the attractivity of females to males. Attractivity increased rapidly in the first few minutes and stabilized after 15–20 min with considerable variations. Experiment II showed that male activity increased in the presence of a female stimulus. One can speculate that location of the female is related to increased movement rather than to direct attraction. Experiment II also showed that male behavior is altered in the same fashion by alkenes from young virgin females as by live females and whole female extracts. We attribute this to increased movement induced by the stimulus. Perception of the female pheromone also could sensitize the male to visual stimuli and explain the observed exploration of any black spot to determine if it is a female.

Although only one trial was made for each extract, the results of experiment II show that male activity is increased by the presence of young virgin females or a fraction containing the alkenes from young virgin females. Male activity is not affected by the alkane or fatty acid fractions. This outdoor experiment also corroborates the two previous experiments that were performed indoors. The importance of visual stimuli in the final location of a mating partner is confirmed by experiment III. The mate-seeking behavior observed was similar to that reported in numerous Apoidea in the presence of pheromone extracts (Kullenberg, 1973; Barrows, 1975; Tengö, 1979; Cane and Tengö, 1981; Bergmark et al., 1984).

The results of multivariate analysis further support the role of alkenes from female *M. rotundata* acting as a sex pheromone. Discrimination of the young females, older females, and males was based on the relative proportion of alkenes. The main differences involved 9-pentacosene and 7-pentacosene, which were more abundant in young females than in males. A major difference between young and old females was loss of the double bond on the seventh carbon atom.

High amounts of alkenes (exclusively 7-monoenes) have been found in *Bombus appositus* (Hadley et al., 1981). In *Bombus occidentalis*, 9-nonacosene accounts for 22% of cuticular hydrocarbon, but tricosenes and pentacosenes are found in the same proportion as alkanes in *B. protorum*, *B. pascuorum*, and *B. lapidarius* (Oldam et al., 1994). In *B. terrestris* and some species of the *Apis* genus, alkanes are the predominant hydrocarbons, and the most abundant alkenes have more than 30 carbon atoms (Blomquist et al., 1980; McDaniel et al., 1983; Francis et al., 1985, 1989; Carlson et al., 1991). *Nomia nevadensis*, the only solitary bee species that has been chemically analyzed, has no unsaturated hydrocarbons (Hadley et al., 1981). The main difference between the cuticular hydrocarbon composition of *M. rotundata* and other bees is the large alkene proportion (60%) and the number of isomers (37). This is the first time that unsaturated hydrocarbons have been implicated as a sex pheromone in bees (for reviews see Howard, 1993; Singer, 1998). Similar results have been reported in Diptera.

In all previous reports, the attractant properties of females and female

extracts observed in solitary insects have involved species nesting in congregations (Cane and Tengö, 1981). Males in these species are attracted to the nearest female (Raw, 1976), often adopting the same mate-seeking behavior observed in *M. rotundata*. Although this response to olfactory stimulation was rare in our laboratory experiments, it is frequent in natural conditions. Kullenberg (1973) observed this type of behavior in Anthophoridae and Apidae, and authors using methods similar to those of Kullenberg have reported similar findings in other bee families including Colletidae (Cane and Tengö, 1981), Andrenidae (Tengö, 1979), Mellitidae (Bergmark et al., 1984), Halictidae (Barrows, 1975), and Anthophoridae (Alcock et al., 1976). Males in these groups are able to locate females before they emerge (Cane and Tengö, 1981). A possible explanation is that a specific odor may alert males of the same species. However, since only virgin females are selected for mating, it is possible that mate-seeking behavior is simply a response to an interesting odor. Further study is needed to confirm the action of this pheromone in the field.

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DEFOLIATION AFFECTS CHEMICAL DEFENSES IN ALL PLANT PARTS OF RYE SEEDLINGS

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Abstract—The effect of defoliation and consecutive defoliation (condefoliation) of rye seedlings on the allocation patterns of biomass and hydroxamic acids (Hx) was evaluated five days after treatment. Growth of condefoliated seedlings was lower than that of defoliated and nondefoliated ones. Concentration of Hx decreased in shoots of condefoliated seedlings compared to nondefoliated ones, while concentration of Hx in root exudates increased. Allocation of Hx to roots and root exudates increased at the expense of allocation to the shoots in condefoliated seedlings. The ratio of Hx-aglucone to Hx-glucoside was higher in shoots of defoliated and condefoliated seedlings. The decrease in quantity of defense in shoots was accompanied by an increase in its quality, given that aglucones are more toxic than glucosides. The increase in concentration of Hx—an allelopathic compound also involved in mineral uptake by roots—in root exudates of condefoliated seedlings was suggested to lead to an advantage in the acquisition of resources for the regeneration of lost biomass.

Key Words—Defoliation, regrowth, induced responses, chemical defense, hydroxamic acids, rye, *Secale cereale*, Poaceae.

INTRODUCTION

Hydroxamic acids (Hx) are plant secondary metabolites present in wild (Niemeyer et al., 1992; Gianoli and Niemeyer, 1998a) and cultivated (Niemeyer, 1988) Poaceae and are involved in plant resistance to insect herbivores and pathogens (Bergvinson et al., 1995; Niemeyer and Pérez, 1995; Weibull and

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Niemeyer, 1995; Gianoli et al., 1996). In addition, allelopathic properties of Hx have been described in laboratory and field experiments (Barnes and Putnam, 1987; Pérez and Ormeño-Núñez, 1993; Mwaja et al., 1995). Hx are present in all plant parts, and their concentration in shoot, root, and root exudate differs between species and even between cultivars (Argandoña et al., 1981; Pérez and Ormeño-Núñez, 1991; Xie et al., 1991). These compounds occur mainly as glucosides that are transformed to the more biologically active aglucones (Corcuera et al., 1985) by enzymatic hydrolysis upon tissue damage (Hofman and Hofmanová, 1971) . The most abundant Hx in rye (*Secale cereale* L.) is 2-β-*O*-D-glucopyranosyl-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc).

Changes in Hx concentrations as a consequence of insect attack or localized artificial damage simulating it have been widely described (Gutiérrez et al., 1988; Niemeyer et al., 1989; Morse et al., 1991; Gianoli and Niemeyer, 1997, 1998b), while the effect on Hx metabolism of damage by defoliation, a process analogous to grazing or harvesting, has been reported recently in rye (Collantes et al., 1997) and in maize (Collantes et al., 1998). The latter work focused on the effect of defoliation on Hx levels and allocation patterns within the directly affected plant part, i.e., the shoot. The present work addresses the effect of defoliation on Hx levels and allocation patterns between shoots, roots, and root exudates of rye seedlings by determining the concentration of Hx the relative content of Hx (percentage of whole plant content of Hx), and the ratio of Hx-aglucone to Hx-glucoside. These parameters were also determined in seedlings subjected to a consecutive defoliation (hereafter called condefoliation) in order to evaluate the effect of extended damage on the induction of plant responses. In comparisons of nondefoliated and defoliated seedlings, growth after defoliation is considered analogous to growth after germination since both processes involve the production of new leaf tissue.

METHODS AND MATERIALS

Plants. Seeds of *S. cereale* cv. Tetra were obtained from CAMPEX-Baer, Chile, and germinated in individual plastic pots (100 ml) filled with sterilized sand (Anasac). Seedlings were grown in a room at 25 ± 3°C, 16L : 8D photoperiod and were irrigated with a nutrient solution (Anasac) containing N, P, and K (6 : 4 : 3 ratio) and micronutrients. Defoliated seedlings were obtained by cutting the plant just above the coleoptile (removing all shoot material) five days after sowing; condefoliated seedlings by cutting again five days after the first defoliation. Nondefoliated, defoliated, and condefoliated seedlings (seven seedlings per treatment) were evaluated five days after sowing, defoliation, and condefoliation, respectively. The defoliation procedure mimics natural browsing damage in grasses (Belsky, 1992).

Extracts. Seedlings were carefully drawn from the sand to avoid root damage. Roots were washed with distilled water directly onto the pots to remove sand particles. Seedlings were weighed and then shoots and roots were separated and immediately macerated by using a mortar and pestle with ca. 300 mg sea sand in 1 ml 0.1 M glycine HCl buffer, pH 2. Plant parts were macerated immediately in order to avoid spurious conversion of Hx glucosides into aglucones due to the scission of the plant. The weight of roots and shoots of experimental seedlings was not directly determined but rather estimated from weight data from additional groups of seedlings ($N = 7$ each) subjected to the same conditions within each treatment. These seedlings were drawn from the sand, weighed, cut into root and shoot, and finally both roots and shoots were weighed. Linear relationships were established between total plant weight and shoot weight ($R^2 = 0.95$ for nondefoliated; $R^2 = 0.98$ for defoliated; and $R^2 = 0.86$ for condefoliated) and between total plant weight and root weight ($R^2 = 0.96$ for nondefoliated; $R^2 = 0.95$ for defoliated, and $R^2 = 0.73$ for condefoliated). From these relationships and the total weight of experimental seedlings, the independent weights of roots and shoots were estimated. Root–shoot ratios of biomass as well as Hx concentrations were calculated using these values. The sand in the pots was washed with 100 ml distilled water to obtain root exudates. The washing solution was evaporated to dryness under vacuum at 45° C and the dry residue extracted with 1 ml *n*-butanol. All extracts were centrifuged at 10,400*g* for 15 min and the supernatant stored in a freezer until analysis.

Chemical Analysis. A 100-ml aliquot of extracts was directly injected into an HPLC. An RP-100 LiChrospher-C18 column (5 mm ID; Merck) was used with a constant solvent flow of 1.5 ml/min, and the following linear gradients between solvents A (MeOH) and B (0.5 ml 85% H_3PO_4 in 1 liter H_2O): 0–9 min 30% A, 9–11 min 100% A, and 11–15 min 30% A. Detection of compounds eluting from the column was performed at 263 nm. Both DIBOA-glucoside and its aglucone were analyzed. Retention times for aqueous extracts were 2.7 ± 0.1 and 3.7 ± 0.1 min for DIBOA-glucoside and DIBOA-aglucone, respectively, and 1.8 ± 0.1 min for the DIBOA-aglucone in *n*-butanol. Identity of peaks was confirmed by coinjection of standards dissolved in water and *n*-butanol, respectively.

Statistical Analysis. One-way ANOVA was performed to analyze separately: shoot biomass, concentration of Hx, relative content of Hx and Hx aglucone–glucoside ratio. In order to comply with ANOVA assumptions, the following data transformations were performed: square-root for concentration of Hx in root exudates; logarithmic for shoot biomass and for relative content of Hx; and arc-sin for the Hx-aglucone to Hx-glucoside ratio. The concentration of Hx in root exudates was estimated by dividing the content of Hx in root exudates by the fresh weight of roots. A Newman-Keuls test was used to evaluate differences between treatments.

RESULTS

Shoot biomass of seedlings was affected by treatment ($P < 0.001$). Nondefoliated and defoliated seedlings showed no significant differences between them ($P = 0.141$), but both were significantly different from condefoliated ones ($P < 0.001$ for both comparisons) (Table 1). Root biomass of seedlings was not affected by treatment ($P = 0.567$), and root–shoot ratios differed between treatments (Table 1). The statistical significance of the latter differences could not be determined because those were single values, obtained after analyses of regression (see Methods and Materials).

Treatment affected the concentration of Hx in shoots ($P = 0.021$) and root exudates ($P = 0.036$), but not in roots ($P = 0.280$). No significant differences were found in shoots between nondefoliated and defoliated seedlings, nor between defoliated and condefoliated ones. However, nondefoliated and condefoliated seedlings were different (Table 2). Similar relations between nondefoliated, defoliated, and condefoliated seedlings were found in root exudates. However, the concentration of Hx in shoots of condefoliated seedlings was lower than nondefoliated ones, while in root exudates it was higher (Table 2).

On the other hand, the effect of treatment on the relative content of Hx was significant in shoots ($P < 0.001$), roots ($P < 0.001$), and root exudates ($P < 0.001$). No differences were found between nondefoliated and defoliated seedlings, while condefoliated ones were different from these two. These relations between nondefoliated, defoliated, and condefoliated seedlings were the same in shoots, roots, and root exudates. However, the relative content of Hx in condefoliated seedlings was lowest in shoots, and highest in roots and root exudates (Table 3).

The ratio of Hx-aglucone to Hx-glucoside was affected by treatment in shoots ($P = 0.005$) but not in roots ($P = 0.828$). Differences were found in shoots between nondefoliated seedlings and both defoliated and condefoliated ones, the latter two being not significantly different from each other and higher

TABLE 1. BIOMASS OF SHOOT AND ROOT OF RYE SEEDLINGS SUBJECTED TO DEFOLIATION AND CONSECUTIVE DEFOLIATION

	Biomass (mg, mean ± SE)[a]		
	Nondefoliated	Defoliated	Condefoliated
Shoot	103.37 ± 8.66 a	129.18 ± 14.77 a	42.13 ± 4.53 b
Root	155.05 ± 12.99 a	139.95 ± 16.00 a	133.42 ± 14.36 a
R : R[b]	1.50	1.08	3.17

[a]Means followed by the same lowercase letter within rows are not significantly different ($P > 0.05$, Newman-Keuls test).
[b]Ratio of root–shoot biomass.

TABLE 2. CONCENTRATION OF HYDROXAMIC ACIDS IN RYE SEEDLINGS SUBJECTED TO DEFOLIATION AND CONSECUTIVE DEFOLIATION

	Hx conc (mmol/kg fresh wt, mean ± SE)[a]		
	Nondefoliated	Defoliated	Condefoliated
Shoot	19.31 ± 1.48 a	17.13 ± 1.96 ab	12.78 ± 1.11 b
Root	3.93 ± 0.32 a	3.23 ± 0.32 a	3.44 ± 0.28 a
Exudates[b]	0.26 ± 0.03 a	0.64 ± 0.17 ab	0.47 ± 0.04 b

[a]Means followed by the same lowercase letter within rows are not significantly different ($P > 0.05$, Newman-Keuls test).
[b]The concentration of Hx was estimated by dividing the content of Hx in root exudates by the fresh weight of roots.

TABLE 3. RELATIVE CONTENT OF HYDROXAMIC ACIDS (PERCENTAGE OF WHOLE PLANT CONTENT) IN RYE SEEDLINGS SUBJECTED TO DEFOLIATION AND CONSECUTIVE DEFOLIATION[a]

	Nondefoliated	Defoliated	Condefoliated
Shoot	72.41 ± 2.90 a	80.05 ± 2.87 a	50.86 ± 1.78 b
Root	25.98 ± 2.92 a	17.11 ± 3.01 a	43.61 ± 1.89 b
Exudates	1.61 ± 0.12 a	2.84 ± 0.76 a	5.53 ± 0.74 b

[a]Values are means ± SE. Means followed by the same lowercase letter within rows are not significantly different ($P > 0.05$, Newman-Keuls test).

TABLE 4. RATIO OF HYDROXAMIC ACID-AGLUCONE TO HYDROXAMIC ACID-GLUCOSIDE IN RYE SEEDLINGS SUBJECTED TO DEFOLIATION AND CONSECUTIVE DEFOLIATION[a]

	Nondefoliated	Defoliated	Condefoliated
Shoot	0.11 ± 0.01 a	0.18 ± 0.02 b	0.16 ± 0.01 b
Root	0.72 ± 0.12 a	0.69 ± 0.07 a	0.68 ± 0.04 a

[a]Values are means ± SE. Means followed by the same lowercase letter within rows are not significantly different ($P > 0.05$, Newman-Keuls test).

than nondefoliated ones (Table 4). Only the aglucone of DIBOA was found in root exudates.

DISCUSSION

Comparisons between nondefoliated, defoliated, and condefoliated plants are inevitably complicated by the fact that the seedlings under these treatments

differ in chronological age. This might be thought to be critical given that the dynamics of accumulation of Hx in cereals is sensitive to plant age (Argandoña et al., 1981). Nonetheless, previous results on rye seedlings with identical experimental procedures showed that the dynamics of defoliated plants does not follow the typical chronological pattern of Hx accumulation (Collantes et al., 1997). This indicates that the confounding effects of seedling age are overwhelmed by the treatment effects. Therefore, considering that there is no way to uncouple age and regrowth after defoliation, and for the sake of allowing comparisons, growth after defoliation is considered here as analogous to growth after germination, since both processes involve the production of new leaf tissue.

Research on plant responses induced by insect feeding or wounding has focused mainly on the increase in concentration of defensive compounds (Tallamy and Raupp, 1991). However, plants may respond to herbivore attack or artificial damage in a variety of ways. We considered that defoliation of rye seedlings may trigger responses at three different levels: (1) an increase in the concentration of Hx in any plant part (including root exudates); (2) changes in the allocation patterns of Hx among shoot, roots, and root exudates; and (3) qualitative changes in chemical defense by transformation of Hx-glucosides into the more toxic aglucones.

The concentration of Hx in plant parts was only affected in shoots and root exudates of condefoliated seedlings. However, while the Hx concentration of shoots decreased in condefoliated seedlings, that of root exudates increased, and hence constitutes an induced response (*sensu* Karban and Myers, 1989) upon defoliation. In this case, the plant compensated for loss of photosynthetic biomass—and hence a likely decrease in fitness—through the increased exudation of an allelopathic compound that could enhance its competitiveness vis-à-vis neighboring plants. The role of rye-exuded Hx in allelopathy has been widely substantiated (Barnes and Putnam, 1987; Pérez and Ormeño-Núñez, 1991, 1993; Mwaja et al., 1995). The fact that condefoliated rye seedlings show this effect, but defoliated ones do not, suggests that this response positively correlates with intensity of damage, as would be expected for an induced response (Karban, 1991; Zangerl and Bazzaz, 1992). However, given that Hx data of defoliated seedlings showed an unexpectedly high dispersion, leading to a lack of significant differences with the other treatments, it cannot be ruled out that rye seedlings also could respond to an initial defoliation by increasing Hx exudation.

The decrease in the shoot of Hx relative content observed in condefoliated seedlings could be a consequence of the decrease in relative shoot biomass in that treatment, as is evident from the root–shoot ratio and the shoot data. This might be envisioned as a match between biomass allocation and the defensive endowment of a plant part. However, data of root biomass and relative content of Hx challenge the generality of such an assertion. While the root biomass did not differ between treatments, roots of condefoliated seedlings had a higher relative content of Hx.

The higher allocation of Hx to roots and root exudates in condefoliated seedlings may be attributed to the role of below-ground parts in resource acquisition for regeneration of lost tissue. Interestingly, a role in iron uptake of root-exuded Hx has been reported (Pethó, 1993). Moreover, the increase in Hx exudation by condefoliated seedlings apparently occurred at the expense of Hx allocation to the shoot. This quantitative loss of defense in shoots may be compensated for by the production of qualitatively enhanced defenses through the transformation of DIBOA-Glc into its aglucone. Aglucones have been reported to have enhanced toxicity to herbivorous insects with respect to Hx glucosides (Corcuera et al., 1985). There is, indeed, a higher aglucone–glucoside ratio in the shoots of condefoliated seedlings, in which Hx concentration decreased, but not in the roots, where Hx concentration remained constant.

The present work emphasizes that induced plant responses to defoliation are not restricted to shoots. Condefoliation brought an increase in the allocation of Hx to roots and root exudates, probably due to an increase in their relative importance for plant fitness due to the initial loss of aerial biomass and the subsequent subcompensatory growth. Moreover, the increase in the exudation of Hx, compounds implicated in allelopathy, could lead to an advantage in the acquisition of resources for the regeneration of lost biomass if it negatively affects neighboring plants of other species. Autotoxicity by Hx does not occur (Niemeyer and Pérez, 1995). In addition, defoliation improved the quality of the chemical defense through the transformation of Hx-glucosides into more toxic aglucones. This response is somewhat analogous to cyanogenesis induced by herbivory in plants containing cyanogenic glycosides (Dirzo and Harper, 1982; Seigler, 1991).

Provided that mature wild Poaceae show responses to defoliation similar to those of rye seedlings, and given that Hx inhibits germination and seedling growth of several species (Wolf et al., 1985; Barnes and Putnam, 1987; Pérez, 1990; Chase et al., 1991), it is tempting to speculate that the increase in root exudation of Hx after natural defoliation [e.g., browsing by goats (Klansek et al., 1995) or hares (Wolfe et al., 1996)] could affect the distribution of plant species in grazed grassland communities (see Belsky, 1992). Further evaluation in natural systems of the actual benefits of these induced responses is needed to clarify the role of both natural (mammal grazing) and artificial (harvesting practices) defoliation on the ecology of rye plants. Hypothetical benefits should be evaluated mainly in terms of resistance to herbivory and plant–plant interactions.

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SIMULTANEOUS DISRUPTION OF PHEROMONE COMMUNICATION IN *Choristoneura rosaceana* AND *Pandemis limitata* WITH PHEROMONE AND ANTAGONIST BLENDS

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Abstract—In British Columbia, trapping and wind-tunnel studies demonstrated that (*Z*)-9-tetradecenyl acetate (Z9–14 : OAc), a minor component of the sex pheromone for *Pandemis limitata*, acted as a pheromone antagonist to a sympatric species, *Choristoneura rosaceana*. Addition of >1% Z9–14 : OAc to the four-component *C. rosaceana* pheromone in a wind tunnel resulted in significant reductions in the proportion of male *C. rosaceana* that wing fanned, locked on to the plume in flight, oriented upwind, and made source contact, compared to the responses to the pheromone alone. Disruption of pheromone communication was tested in 33.3 × 33.3-m plots, at a release rate of 10 mg/ha/hr using Conrel fiber dispensers. Z9–14 : OAc applied alone did not disrupt orientation to virgin-female-baited traps for either *C. rosaceana* or *P. limitata*. A 1 : 1 mixture of Z9–14 : OAc and the four-component *C. rosaceana* pheromone was as effective as the pheromone alone at disrupting orientation of *C. rosaceana* males to virgin-female-baited traps, demonstrating that disruption apparently did not occur through false-trail following. The 1 : 1 mixture of Z9–14 : OAc and the *C. rosaceana* pheromone also reduced catches of *P. limitata* males in virgin-female-baited traps, but not significantly more than the 83% disruption caused by the pheromone alone. Therefore, the *C. rosaceana* pheromone could be used alone or with Z9–14 : OAc to disrupt communication and, presumably, mating in both leafrollers simultaneously.

Key Words—*Choristoneura rosaceana*, *Pandemis limitata*, Tortricidae, leafrollers, communication disruption, sex pheromone, pheromone antagonist.

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INTRODUCTION

Two sympatric leafrollers, the oblique-banded leafroller, *Choristoneura rosaceana* (Harris), and the three-lined leafroller, *Pandemis limitata* (Robinson), are moderately important pests of pome fruits in the Okanagan and Similkameen valleys of British Columbia. Implementation of autocidal control of the codling moth, *Cydia pomonella* L (Dyck et al., 1993), will likely cause these species to become more serious pests (Judd et al., 1991). This will require noninsecticidal management if growers are to produce pesticide-reduced or organically grown fruit.

Both species depend primarily on female-produced sex pheromones for mate finding and share the same major pheromone component, (*Z*)-11-tetradecenyl acetate (Z11–14 : OAc) (Roelofs et al., 1976; Vakenti et al., 1988). Minor pheromone components for *C. rosaceana* in western North America are (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl alcohol, and (*Z*)-11-tetradecenyl aldehyde (Vakenti et al., 1988). (*Z*)-9-tetradecenyl acetate (Z9–14 : OAc) is a minor component for *P. limitata* (Roelofs et al., 1976).

Pheromone-based mating disruption has been used successfully to control several species of insect pests (Cardé and Minks, 1995), but there are few examples of multiple-species mating disruption systems (Ridgway et al., 1990). Sympatry of these and other leafroller species in British Columbia demands an integrated approach to the development of a multiple-species mating disruption system. One approach to achieving this is the use of common pheromone components (Deland et al., 1994; Pfeiffer et al., 1993; van Deventer and Blommers, 1992) but another might be the use of pheromone components that act interspecifically as antagonists. Although pheromone antagonists alone have not been effective mating disruptants (e.g., Daterman et al., 1975; Kaae et al., 1974), they can stimulate cross-species attraction (Stadelbacher et al., 1983). A combination of pheromone components and antagonists could result in mating disruption by causing species to emigrate from treated areas (Bengtsson et al., 1994). Dodecyl acetate was shown to reduce pheromone trap capture of male *C. rosaceana* in New York (Roelofs and Comeau, 1971). Cardé and Baker (1984) cite unpublished evidence from eastern North America that Z9–14 : OAc, the minor pheromone component in *P. limitata*, can inhibit attraction of *C. rosaceana* to its pheromone.

We tested the hypothesis that Z9–14 : OAc is a pheromone antagonist for a western North American population of *C. rosaceana* and measured the efficacy of Z9–14 : OAc alone and in combination with the sex pheromone of *C. rosaceana* as disruptants of pheromone communication in both species.

METHODS AND MATERIALS

Wind-Tunnel Description. We used a wind tunnel built from the design of Miller and Roelofs (1978). Air was pushed through the tunnel, and the

pheromone plume was drawn downwind by a centrally located, variable-speed exhaust fan. The flight section of the tunnel was 2.45 m long and 1 m high at its center. Uniform, dim white light (0.5 lux) was provided by six, 25-W incandescent bulbs, shielded by sheet metal and held 17 cm above three layers of fine black fibreglass mesh (Bay Mills, Mississauga, Ontario) and two layers of white templast (Cameron Ashley Building Products, Kelowna, British Columbia). Tunnel temperatures varied between 22 and 26.5°C. A wind speed of 0.35–0.40 m/sec was maintained.

Wind-Tunnel Experimental Protocol. Male *C. rosaceana* pupae were obtained from a 1-year-old laboratory colony maintained on a pinto bean-based diet at 24°C and a 16L : 8D photoregime. Pupae were placed individually in 150-ml clear, plastic cups provided with water in a cotton dental wick. Cups containing pupae were held at 24°C under a reversed 8D : 16L photoregime and were isolated from adult females to ensure no exposure to pheromone. One- to 3-day-old male moths were collected during the last half hour of the photophase and cooled for 10–30 min at 0.5°C. Immobile moths were placed in cylindrical wire mesh release cages (3–4 cm diam. × 1.5 cm height) attached to 5- × 5-cm wire mesh bases equipped with removable sheet metal lids. Males were evenly distributed among treatments by age to avoid any age-related bias in behavior. Before testing, all males in release cages were transferred to the wind-tunnel room where they acclimated to ambient conditions during the first 0.5 hr of scotophase.

Bioassays were conducted during the next 2 hr of scotophase, the peak period of response to pheromone (Knight et al., 1994). The order of presentation of pheromone treatments was randomly determined. Rubber septa lures (The West Company, Linville, Pennsylvania) were hung from a wire extending below a metal ring stand, 44 cm above the tunnel floor and 34 cm from its upwind end. Five to seven moths were presented individually to each pheromone treatment. A screen cage containing a male was placed on a mobile release device through a port in the side of the tunnel and pushed into the plume by a lever operated from outside the tunnel. The sheet metal lid was then lifted from the cage by pulling on an attached line, also operated outside the tunnel. When in the plume, moths were 43 cm above the tunnel floor and 56 cm from the downwind end, allowing for a 1.5-m flight distance from release point to source. Individual male response to each pheromone treatment was graded as yes or no for the following behaviors: wing fanning, take-off, locking on to the plume, oriented upwind flight, and source contact. The release device and ring stand were rinsed with hexane between each treatment. Between replicates both cages and lids were heated for 4 hr at 200°C; lids were rinsed in 95% ethanol.

Experiments 1–3: Wind-Tunnel Experiments. Three wind-tunnel experiments were conducted to investigate the hypothesis that addition of Z9–14 : OAc to the *C. rosaceana* pheromone would inhibit the response of male *C. rosaceana*.

Experiment 1 compared the attractiveness of rubber septa lures containing 100 μg of pheromone in 37 μl of HPLC-grade hexane to lures containing 100 μg of the same blend plus 50, 6, and 1 μg of Z9–14 : OAc added in 10 μl of HPLC-grade hexane. The *C. rosaceana* pheromone contained a 100 : 2 : 1 : 1.5 ratio [confirmed by gas chromatographic (GC) analysis] of Z11–14 : OAc, E11–14 : OAc, Z11–14 : Ald, and Z11–14 : OH (Thomson et al., 1991; Vakenti et al., 1988). Purities of the compounds used were: Z11–14 : OAc, 98% with 2% E isomer; Z11–14 : Ald, 96%; Z11–14 : OH, 97%; and Z9–14 : OAc, 96%.

Experiment 2 was identical to experiment 1 except that 100-μg lures of the complete pheromone were compared to lures containing 100 μg of the same blend plus 1, 0.1, and 0.01 μg of Z9–14 : OAc added in 10 μl of HPLC-grade hexane. Experiment 3 tested the positive control, 100 μg of the *C. rosaceana* pheromone, septa containing 100 μg of the same blend plus 1 μg Z9–14 : OAc, septa loaded with 1 μg Z9–14 : OAc alone, and negative control septa loaded with HPLC grade hexane alone. All treatments in experiment 3 were diluted in 47 μl of HPLC-grade hexane. Loaded septa were aged in a fume hood for 24 hr at 22°C, stored in a freezer at 15°C until use, and warmed in a fume hood at 22°C for 1 hr.

Experiment 4: Trapping Experiment. Experiment 4 was designed to test if Z9–14 : OAc could prevent male western *C. rosaceana* from locating synthetic pheromone sources under field conditions. Pheromone blends tested included: (1) the *C. rosaceana* pheromone (Thomson et al., 1991; Vakenti et al., 1988); (2) Z9–14 : OAc alone; and (3) a 94 : 6 blend of the four-component *C. rosaceana* pheromone plus Z9–14 : OAc. The blends for treatments 1 and 3 were loaded onto red rubber septa lures at a dose of 3 mg in 200 μl of HPLC-grade hexane. Z9–14 : OAc (treatments 2 and 3) was loaded at a dose of 0.18 mg. Septa were separated by treatment and transported to field sites in glass jars housed in refrigerated containers. Purities of compounds used were as in experiments 1–3.

Traps were constructed from opposing wing-trap tops (Phero Tech Inc., Delta, British Columbia) spaced 5 cm apart. Stickem Special (Phero Tech) was thinly applied to the inside surface of the trap bottom (an inverted top) to capture male moths. At the study site, lures were suspended from the inside top of traps with a stick pin. Traps were hung by a wire hanger approximately 1.5 m high on the north side of apple trees. They were placed 25 m apart in three randomized complete blocks, two in an orchard growing McIntosh apples, and one in an orchard approximately 1 km distant growing Red and Golden Delicious apples. Three replicates were run in each of four, two-night periods between August 10 and 27, 1994, for a total of 12 replicates. Trap positions were rerandomized between replicates, and new lures were used in each replicate.

Experiment 5: Communication Disruption Experiment. The efficacy of using Z9–14 : OAc alone and in combination with the *C. rosaceana* pheromone as an atmospheric treatment to disrupt pheromone communication of *C. rosa-*

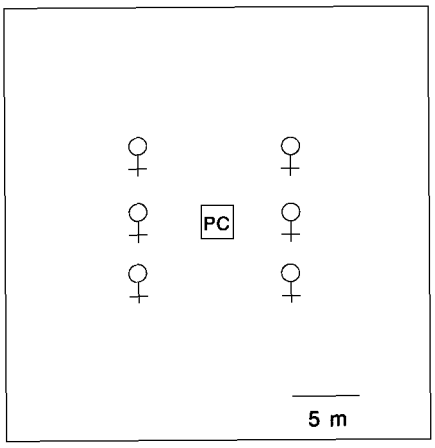

FIG. 1. Field plot design used to test disruption of pheromone communication in experiment 5. PC = plot center; ♀ = position of four female-baited traps, two baited with *C. rosaceana* and two with *P. limitata;* one trap for each species at 1.8 m and at 2.8–2.9 m heights on opposite sides of a tree.

ceana and *P. limitata* in the field was tested in a small-plot protocol (Figure 1) from August 18 to September 6, 1995. Four 0.1-ha plots were established in two orchards in Cawston, British Columbia that grew organic Red Delicious, Gala, and Spartan apples with tree-row spacings, heights, and tree widths described in Table 1. Plots were at least 40 m apart and at least 10 m from the edge of the orchard. Atmospheric semiochemical treatments were assigned to these plots following a Latin-square design and rerandomized so that each treatment occupied each plot for a maximum of 14 days. The four treatments included: (1) the four-component *C. rosaceana* sex pheromone; (2) *Z*9–14 : OAc alone; (3) treatments 1 + 2 in a 1 : 1 ratio; and (4) a nontreated control.

Atmospheric semiochemical treatments were applied with Conrel fiber tape dispensers (Ecogen Inc., Billings, Montana). In each plot, 100 tapes (1000/ha) each with 100 fibers were hung in the upper third of the tree canopy on the north side of the tree, providing an approximate release rate of 10 mg/ha/hr, at 20°C (Ecogen). The actual composition of pheromone components in Conrel fiber dispensers in treatment 1 (the four-component *C. rosaceana* pheromone) was determined by gas chromatographic analysis to be a 90.8 : 4.2 : 3.3 : 1.7 ratio of *Z*11–14 : OAc, *E*11–14 : OAc, *Z*11–14 : OH, and *Z*11–14 : Ald; that for treatment 2 (*Z*9–14 : OAc) was a 97.8 : 2.2 ratio of *Z*9–*Z*9–14 : OAc and *E*9–14 : OAc. In treatment 3, fiber-tape disruption dispensers with 100 fibers of the *C. rosaceana* pheromone were attached to dispensers containing 100 fibers of *Z*9–14 : OAc with a release rate of 10 mg/ha/hr from each dispenser type. New dispensers

TABLE 1. DESCRIPTION OF PLOTS USED FOR EXPERIMENT 5, DISRUPTION OF COMMUNICATION IN *C. rosaceana* AND *P. limitata* IN SIMILKAMEEN VALLEY

Plot	Apple variety	Tree spacing (m, tree × row)	Tree height (m, $\bar{X} \pm$ SE) $N = 10$	Tree width (m, $\bar{X} \pm$ SE)[a] $N = 10$
1	Red Delicious	4 × 5.7	4.23 ± 0.21	3.95 ± 0.13
2	Red Delicious	4 × 5.7	4.49 ± 0.19	4.06 ± 0.10
3	Gala	3.4 × 5.6	3.31 ± 0.23	2.91 ± 0.31
4	Spartan	5.6 × 6.2	4.72 ± 0.19	4.89 ± 0.20

[a]Distance between longest branch tips.

were deployed at the beginning of each replicate, at least 48 hr before assessing the effect of atmospheric treatments on communication disruption.

Disruption caused by semiochemical treatments was assessed with wing traps (as described) baited with virgin females of either species obtained from laboratory colonies. Pupae were sexed, and females were placed individually in 150-ml cups and provided with a water source. Cylindrical mesh cages (3 cm diam. × 5 cm height) with a water source, each containing a ≤4-day-old virgin female, were transported to the field in refrigerated containers and suspended inside wing traps with push pins. Traps for each species were hung in the tree canopy at both high (2.8–2.9 m) and low (1.8 m) positions. Traps were placed in the plot center in six groups of four traps (Figure 1) with one female-baited trap for each of the two species at each of the two heights on opposing sides of each tree. On each of two days for each replicate, traps were baited in late afternoon, and catches were recorded the following morning. The proportion of female-baited traps catching at least one conspecific male in each atmospheric treatment was calculated as a measure of the disruptive effect relative to the nontreated control. To avoid any possible contamination, trap wires and traps were used with only one semiochemical treatment and were moved between replicates.

Statistical Analyses. Proportional data were generated in nearly all experiments. Levesque (1990) demonstrated that logistic regression is a more powerful method of analyzing proportional data than either the classical ANOVA on arcsin transformed data or χ^2 analysis (Zar, 1984). Given that the possibility of committing a type II error (not rejecting the null hypothesis when it is false) is reduced when the most powerful type of analysis is employed (Zar, 1984), proportional data were analyzed by logistic regression models (GLIM, 1985).

A logistic regression model is a generalized linear model used to determine the relationship between a binomially distributed response variable (p_i), of the form y/n, where y is the number of successes, e.g., traps catching at least one male out of N, the total number tested, and one or more explanatory variables x_i. The logistic regression model used has the form:

$$p_i = \exp(\beta_0 + \beta_1 x_{1i} + \ldots \beta_k x_{ki})/1 + \exp(\beta_0 + \beta_1 x_{1i} + \ldots \beta_k x_{ki})$$

where the relationship between p_i and the x_i is sigmoidal, but it can be shown (Collett, 1989) that the logit (p_i) of this function is linearly related to x_i,

$$\text{logit}\,(p_i) = \log[p_i/(1 - p_i)] = \beta_0 + \beta_1 x_{1i} + \ldots \beta_k x_{ki}$$

In experiments 1–3 proportions of male *C. rosaceana* responding in the wind tunnel to sex pheromone alone and with varying quantities of Z9–14 : OAc added were compared by a linear logistic regression model (GLIM, 1985) in which blocks of time or replicates and quantities of Z9–14 : OAc were treated as explanatory variables. In experiments 1–3, a categorical model was used, including experiments 1 and 2 where the dose of Z9–14 : OAc added to the pheromone could be considered as a continuous variable. This approach was taken in these cases because the dose of Z9–14 : OAc required to inhibit the *C. rosaceana* response was of interest, and this point could only be identified if a categorical model were used. Fitting of the logistic regression model was followed by multiple Z tests to compare individual proportions similar to a multiple comparisons test on means. The α value was adjusted to control for type I error which depends on the number of comparisons being conducted (Zar, 1984).

Experiment 4, which examined the effect of adding Z9–14 : OAc to synthetic sex pheromone in wing traps in the field provided such unequivocal results that no statistical analysis was necessary.

In experiment 5, the efficacy of atmospheric semiochemical treatments as communication disruptants was assessed by comparing the proportion of virgin-female-baited traps capturing at least one conspecific male. These proportions were compared with a linear logistic regression model with disruption treatments, replicates, and plots coded as categorical variables (GLIM, 1985), followed as above by Z tests to compare the treatment proportions by using adjusted α values (Zar, 1984).

The effect of trap position on the capture of male moths within disruption plots was analyzed by using a split-plot ANOVA, with pheromone treatment, plot, and replicate as whole-plot factors and trap height as the subplot factor (SAS, 1988). Replicate and plot were specified as random factors. Numbers of males captured were transformed by $\ln(x + 1)$. Except where otherwise indicated, $\alpha = 0.05$.

RESULTS

Experiments 1–3: Wind-Tunnel Experiments. In experiment 1, in the wind tunnel, departure from the release device was not influenced by the addition of Z9–14 : OAc (Figure 2, Table 2), but in the presence of 1–50 μg of Z9–14 : OAc,

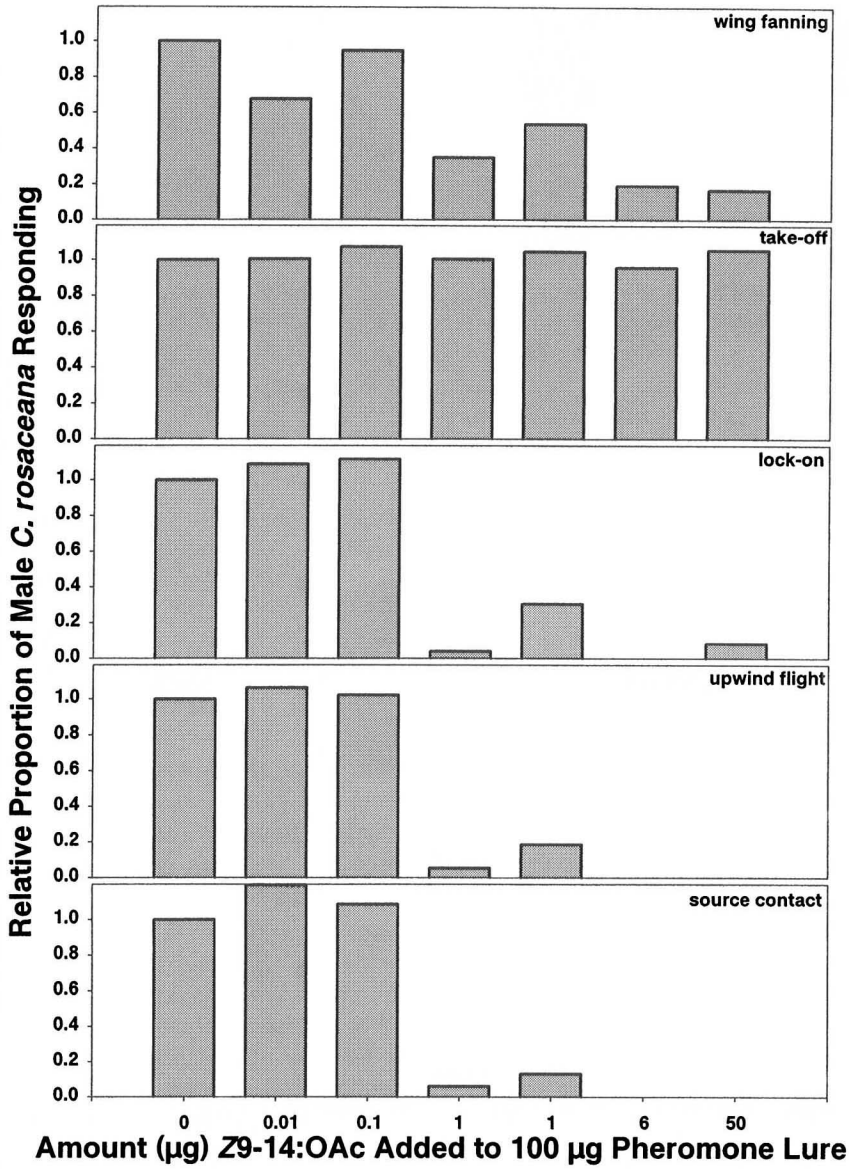

FIG. 2. Relative proportions (proportion of *C. rosaceana* responding to *C. rosaceana* pheromone = 1.0) of male *C. rosaceana* responding in a wind tunnel in experiments 1 and 2 to 100 μg of its sex pheromone and 100 μg of pheromone with increasing quantities of Z9–14 : OAc added (N = 7).

TABLE 2. RESPONSE BY *C. rosaceana* MALES IN EXPERIMENTS 1 AND 2 IN A WIND TUNNEL TO *C. rosaceana* PHEROMONE ALONE AND WITH Z9–14 : OAc ADDED[a]

Experiment	Behavior	Z9–14 : OAc added (μg) to 100 μg pheromone[b]	Proportion of *C. rosaceana* males exhibiting response[c]	Logistic regression standard errors Mean proportion +SE	−SE
1	wing fanning	0	0.80a	0.86	0.74
1		1	0.41b	0.49	0.34
1		6	0.15c	0.21	0.10
1		50	0.13c	0.19	0.09
1	take-off	0			
1		1	no treatment effect		
1		6			
1		50			
1	lock-on	0	0.52a	0.60	0.44
1		1	0.16b	0.23	0.11
1		6	0	*d*	*d*
1		50	0.04b	0.09	0.02
1	upwind flight	0	0.37a	0.44	0.30
1		1	0.07b	0.12	0.04
1		6	0	*d*	*d*
1		50	0	*d*	*d*
1	source contact	0	0.35a	0.42	0.28
1		1	0.05b	0.09	0.02
1		6	0	*d*	*d*
1		50	0	*d*	*d*
2	wing fanning	0	0.93a	0.96	0.88
2		0.01	0.64b	0.72	0.55
2		0.1	0.89a	0.93	0.83
2		1	0.30c	0.39	0.23
2	take-off	0			
2		0.01	no treatment effect		
2		0.1			
2		1			
2	lock-on	0	0.49a	0.57	0.41
2		0.01	0.53a	0.61	0.45
2		0.1	0.55a	0.62	0.46
2		1	0.02b	0.06	0.01
2	upwind flight	0	0.40a	0.48	0.33
2		0.01	0.43a	0.50	0.35
2		0.1	0.41a	0.49	0.34
2		1	0.02b	0.06	0.01
2	source contact	0	0.36a	0.43	0.29

TABLE 2. CONTINUED

Experiment	Behavior	Z9–14 : OAc added (μg) to 100 μg pheromone[b]	Proportion of *C. rosaceana* males exhibiting response[c]	Logistic regression standard errors Mean proportion +SE	−SE
2		0.01	0.43a	0.50	0.36
2		0.1	0.39a	0.46	0.32
2		1	0.02b	0.06	0.01

[a]Seven replications per experiment with 5–7 males tested per treatment, per replicate.
[b]*C. rosaceana* pheromone source was a 100-μg lure containing a 100 : 2 : 1 : 1.5 mixture of Z11–14 : OAc, *E*11–14 : OAc, Z11–14 : Ald, and Z11–14 : OH.
[c]Proportions followed by the same letter within the same experiment and behavior are not significantly different ($P > 0.05$, Z test).
[d]Standard errors could not be estimated by the logistic regression function.

more males generally flew out of the plume immediately after take-off than normally did in the presence of their pheromone blend. Male *C. rosaceana* were completely deterred from contacting a 100-μg source of its normally attractive pheromone when Z9–14 : OAc was added at rates of 6 and 50 μg (Figure 2). The addition of 1, 6, and 50 μg of Z9–14 : OAc to 100 μg of the sex pheromone also resulted in a significant reduction in the proportion of males wing fanning, locking on to the plume, achieving oriented upwind flight, and source contact (Figure 2, Table 2).

In experiment 2, the addition of 1 μg Z9–14 : OAc significantly reduced the proportion of males that conducted all behavioral responses (Table 2). Not until the quantity of Z9–14 : OAc was ≤0.1 μg did inhibition of pheromone response disappear (Figure 2, Table 2). As in experiment 1, additions of 0.01, 0.1, or 1 μg of Z9–14 : OAc had no influence on take-off from the release device (Figure 2, Table 2).

In experiment 3, an equal proportion of males presented with 1 μg of Z9–14 : OAc alone or with hexane alone departed from the release device (Table 3). A greater proportion of males presented with the sex pheromone alone or with 1 μg of Z9–14 : OAc incorporated into the pheromone departed from the release device than males presented with 1 μg of Z9–14 : OAc alone or hexane alone (Table 3).

Experiment 4: Trapping Experiment. The addition of 6% Z9–14 : OAc to the sex pheromone of *C. rosaceana* resulted in a complete inhibition of conspecific male catches (Figure 3). In turn, *P. limitata* males responded exclusively

TABLE 3. RESPONSE BY *C. rosaceana* MALES IN EXPERIMENT 3 IN A WIND TUNNEL TO *C. rosaceana* PHEROMONE ALONE OR WITH Z9–14:OAc, Z9–14:OAc ALONE, AND HEXANE ALONE[a]

Behavior	Stimulus[b]	Proportion of *C. rosaceana* males exhibiting response[c]	Logistic regression standard errors, Mean proportion +SE	−SE
Wing fanning	*C. rosaceana* pheromone	0.89a	0.93	0.83
	C. rosaceana pheromone + Z9–14 : OAc	0.48b	0.55	0.40
	Z9–14 : OAc	0.09c	0.15	0.06
	Hexane	0	*d*	*d*
Take-off	*C. rosaceana* pheromone	0.86a	0.92	0.78
	C. rosaceana pheromone + Z9–14 : OAc	0.93a	0.97	0.86
	Z9–14 : OAc	0.52b	0.62	0.43
	Hexane	0.51b	0.61	0.42
Lock on	*C. rosaceana* pheromone	0.41a	0.48	0.34
	C. rosaceana pheromone + Z9–14 : OAc	0.07b	0.12	0.04
	Z9–14 : OAc	0	*d*	*d*
	Hexane	0	*d*	*d*
Upwind flight	*C. rosaceana* pheromone	0.27a	0.34	0.21
	C. rosaceana pheromone + Z9–14 : OAc	0.02b	0.06	0.01
	Z9–14 : OAc	0	*d*	*d*
	Hexane	0	*d*	*d*
Source contact	*C. rosaceana* pheromone	0.20a	0.27	0.15
	C. rosaceana pheromone + Z9–14 : OAc	0.02b	0.06	0.01
	Z9–14 : OAc	0	*d*	*d*
	Hexane	0	*d*	*d*

[a]Seven replications of 5–7 males tested per treatment, per replicate.
[b]*C. rosaceana* pheromone source was a 100-μg lure containing a 100 : 2 : 1 : 1.5 blend of Z11–14 : OAc, E11–14 : OAc, Z11–14 : Ald, and Z11–14 : OH. Z9–14 : OAc was tested at a dose of 1 μg.
[c]Proportions followed by the same letter within the same behavior are not significantly different ($P > 0.05$, Z test).
[d]Standard errors could not be estimated by the logistic regression function.

to this altered blend. As expected, Z9–14 : OAc alone did not attract males of either leafroller species.

Experiment 5: Communication Disruption Experiment. In an orchard atmospherically treated with Z9–14 : OAc alone, traps baited with virgin females of either species were as effective at capturing conspecific males as female-baited traps in untreated plots (Figure 4). In contrast, treatment of the atmosphere with the four-component sex pheromone of *C. rosaceana* resulted in 88% and 83% reductions in catches of male *C. rosaceana* and *P. limitata*, respectively. A mix-

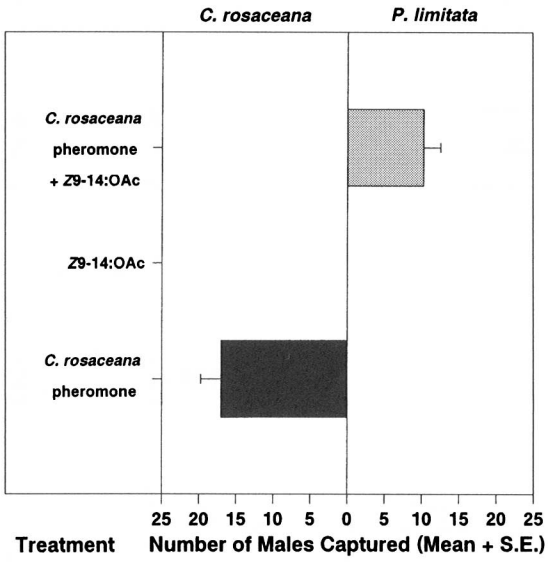

FIG. 3. Mean numbers of male *C. rosaceana* and/or *P. limitata* males captured in wing traps in experiment 4, August 10–27, 1994, at Cawston, British Columbia (N = 12).

ture of Z9–14 : OAc plus the four component sex pheromone in a 1 : 1 ratio did not further reduce the trap catches for either species. Catches of heterospecific males by traps baited with either *C. rosaceana* or *P. limitata* females occurred most frequently in plots treated with Z9–14 : OAc. Experiments examining the importance of Z9–14 : OAc as a reproductive isolating mechanism between these species are in progress. Trap height had no effect on the trap captures of either *C. rosaceana* (F = 0.3246, df = 1,12, P = 0.5794); or *Pandemis limitata* (F = 0.0349, df = 1,12, P = 0.8550).

DISCUSSION

Our results uphold the hypothesis that Z9–14 : OAc acts as a pheromone antagonist for western North American populations of *C. rosaceana*. Despite the fact that eastern and western populations of *C. rosaceana* use slightly different pheromone blends (Thomson et al., 1991; Vakenti et al., 1988), Z9–14 : OAc appears to act without geographic specificity throughout North America. This is possibly due to the sympatry of *C. rosaceana* and *P. limitata*, as well as other orchard-inhabiting tortricid leafrollers, for which Z9–14 : OAc also serves as a pheromone component (Cardé and Baker, 1984). Linn and Roelofs (1995) suggested that pheromone antagonists have evolved to prevent interspecific mat-

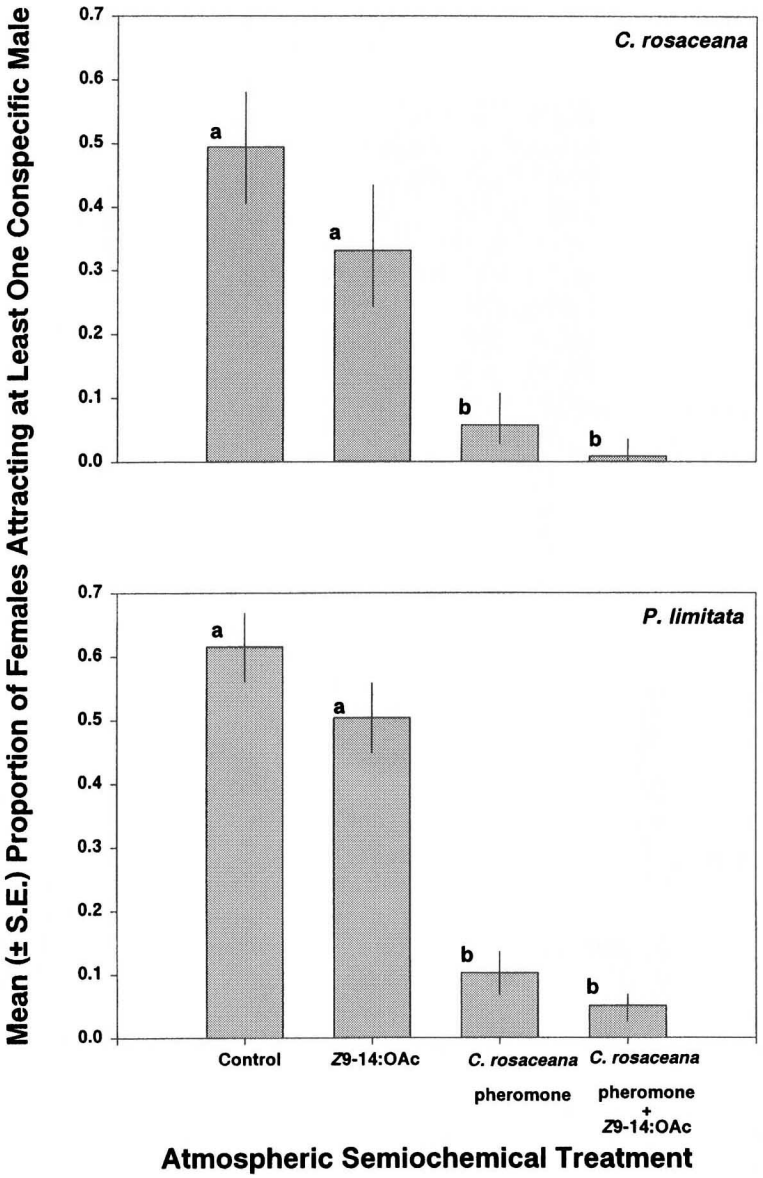

FIG. 4. Proportions of wing traps baited with virgin female *C. rosaceana* or *P. limitata* attracting at least one conspecific male (±SE) in various atmospheric semiochemical treatments in experiment 5, August 18–September 6, 1995 ($N = 4$). Proportions for each species with the same letter are not significantly different, Z test, $P > 0.05$.

ings and increase the uniqueness of the already functional pheromone signal. In fact, research has shown that there are antennal receptors specifically tuned to pheromone antagonists (Christensen et al., 1990; den Otter and van der Haagen, 1989; Grant et al., 1988), and specific glomeruli for processing information about pheromone antagonists are present in the macroglomerular complex of some insects (Mustaparta, 1996). The low concentration (approximately 1%) at which Z9–14 : OAc retains behavioral activity supports the hypothesis that it is an important natural semiochemical.

It has been shown for other insects (Liu and Haynes, 1992; Rumbo et al., 1993) that pheromone antagonists are effective inhibitors of pheromone response only when incorporated into the pheromone blend. Our results support this conclusion. When added to the pheromone of *C. rosaceana*, Z9–14 : OAc caused a significant reduction in pheromone-mediated flight behaviors and completely inhibited trap catches. However, when Z9–14 : OAc was released as a background atmospheric treatment, it had almost no effect on capture of males in female-baited traps. This latter finding is in agreement with other studies on mating disruption attempts that use pheromone antagonists (Daterman et al., 1975; Kaae et al., 1974).

Interestingly, the disruptive effect of the attractive pheromone of *C. rosaceana* was no greater than that of a 1 : 1 mixture of this pheromone and Z9–14 : OAc against male *C. rosaceana*. Suckling and Burnip (1996) also found no difference in disruption of mate location of *Planotortrix octo* (Dugdale) when an attractive pheromone disruptant treatment and an unattractive partial pheromone containing an antagonist were compared. Flint and Merkle (1983) also demonstrated that disruption of mating in the pink bollworm, *Pectinophora gossypiella* (Saunders) was equivalent when using unattractive single pheromone components or the complete pheromone blend. These authors suggested that different mechanisms of communication disruption (Bartell, 1982) were evoked by the different treatments. Several possible mechanisms have been proposed (Bartell, 1982; Cardé, 1990). These include: (1) neurophysiological effects, in which exposure to pheromone causes adaptation of olfactory receptors and/or habituation of olfactory processing at the central nervous system level, resulting in insects being unable to locate natural sources of pheromone or causing shifts in the response to natural pheromone blends; (2) camouflage of the pheromone plume by masking the plume with synthetic pheromone such that searching insects can not distinguish the natural plume from the odor-laden background; and (3) false-trail following, in which insects follow the synthetic pheromone plumes instead of the natural plume.

False-trail following by male *C. rosaceana* may or may not occur in the presence of its four-component pheromone released from point sources at 10 mg/ha/hr as it was in experiment 5. However, it is highly unlikely that disruption was achieved through false-trail following when Z9–14 : OAc, a pheromone

antagonist, was added to the attractive pheromone. Disruption of capture of male *C. rosaceana* by conspecific females when a 1 : 1 mixture of Z9–14 : OAc and *C. rosaceana* pheromone was used as a disruptant could have occurred by neurophysiological mechanisms or by alternative mechanisms, such as inhibition of female calling, induction of male dispersal out of the treated plots, or some combination thereof. The observation that males in wind-tunnel experiments immediately flew out of an otherwise attractive odor plume, when Z9–14 : OAc was added, suggests that the latter mechanism may be invoked. In support of this hypothesis, male pea moths, *Cydia nigricana* F., were repelled from pheromone-treated fields after isomerization of pheromone components to inhibitory isomers (Bengtsson et al., 1994). The effect of Z9–14 : OAc on the behavior of female *C. rosaceana* remains to be investigated.

Although minor components may be effective alone as mating disruptants (Yashima et al., 1975; Miyashita et al., 1976; Kanno et al., 1978; Stadelbacher et al., 1983), Z9–14 : OAc had no such effect on male *P. limitata* (Figure 4). As with male *C. rosaceana* exposed to Z9–14 : OAc alone, *P. limitata* males may have become habituated to this compound, but response to pheromone plumes produced by female *P. limitata* was not altered. It appears that the major pheromone component of both species, Z11–14 : OAc, is required to provide adequate disruption of *P. limitata*, as mate location was reduced similarly in plots treated atmospherically with the *C. rosaceana* pheromone and a 1 : 1 mixture of the pheromone and Z9–14 : OAc.

From a practical standpoint it would be useful to suppress several insect species using one pheromone formulation. This may be possible in British Columbia orchards, as both the *C. rosaceana* pheromone alone and combined with Z9–14 : OAc provide >83% disruption of mate location for both species. The effect of Z9–14 : OAc on other leafroller species in the Okanagan Valley needs to be determined so that a blend suitable for all four sympatric species can be developed.

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FUNGISTATIC ACTIVITY OF *Ruta graveolens* EXTRACT AND ITS ALLELOCHEMICALS

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Abstract—The biological activity of an extract of *Ruta graveolens* and its allelochemicals [8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP) and 4-hydroxycoumarin] (previously isolated) was evaluated gainst pathogenic fungi: *Fusarium solani*, *Pyrenochaeta lycopersici*, and *Trichoderma viride*. In addition a rue lyophilized extract was also tested against *Penicillium* sp., *Thielaviopsis basicola*, and *Verticillium dahliae*. We determined that 5 g rue lyophilized extract/liter, the lowest concentration tested, inhibited the mycelial growth of *P. lycopersici* and *V. dahliae* by 63.6% and 47%, respectively, with IC_{50} (the concentration required to inhibit growth 50%) values of 4.16 and 6.5 g/liter, respectively. Moreover, the rue lyophilized extract was shown to reduce radial growth of all six fungal species significantly at concentrations ranging from 5.0 to 40.0 g/liter. Mycelial growth of *Penicillium* sp. was promoted at 5.0 g lyophilized rue extract/liter and inhibited at higher concentrations. 4-OH coumarin had no effect on the growth of these fungi at concentrations ranging from 0.32 to 2.60 mg/liter, whereas 8-MOP and 5-MOP inhibited the mycelial growth of *F. solani* and *T. viride* with IC_{50} values of 6 mg/liter (5-MOP) and 27 mg/liter (8-MOP) for both fungi, respectively. A mixture of 5-MOP and 8-MOP, tested at 64.8 mg/liter (as they naturally occur in the crude rue extract) significantly inhibited the growth of all tested fungal species. These results suggest potential role for the rue extract and its allelochemicals in controlling pathogenic fungal infections.

Key Words—5-Methoxypsoralen, 8-methoxypsoralen, 4-hydroxycoumarin, *Ruta graveolens* rue, *Fusarium solani*, *Pyrenochaeta lycopersici*, *Penicillium* sp., *Trichoderma viride*, *Thielaviopsis basicola*, *Verticillium dahliae*, fungistatic activity.

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INTRODUCTION

Herbicides continue to be a key component in most integrated weed management systems to control weeds. Nevertheless, extensive use of synthetic herbicides poses serious threats to the environment and public health. There is great incentive to discover biologically active natural products from higher plants that are better than synthetic agrochemicals and are much safer environmentally. The development of natural products such as herbicides and fungicides and their role in biocontrol of plant disease promises to reduce environmental and health hazards (Kennedy et al., 1995; Rice, 1995). Allelochemicals have been investigated as a possible alternative weed management strategy (Macias, 1995). Biologically active natural products (allelochemicals) are now being employed as herbicides and fungicides and in biocontrol of plant diseases (Einhellig, 1984; Rice, 1985). The objective of our research group is to assay medicinal plants, rich in active principles, for their allelopathic activities. The present study deals with the biological activity of *Ruta graveolens* L. extract and the effect of some synthetic compounds [8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP) and 4-hydroxycoumarin] identified from rue (Aliotta et al., 1994) on the growth of certain soil pathogenic fungi.

METHODS AND MATERIALS

Plant Collection, Extraction, and Lyophilization. Fresh leaves of *Ruta graveolens* L. were collected from plants grown at the Research Institute for Vegetable Crops in Pontecagnano (SA), Italy. Two hundred fifty grams of fresh leaf material were soaked in 1 liter of deionized water at 90°C for 10 min, followed by filtration through Whatman No. 1 filter paper (Zobel and Brown, 1988). The filtrate was lyophilized and powdered to yield 10 mg/ml.

Fungal Isolates. Five pathogenic soil-borne fungi [*Fusarium solani* (Mart.) Appel et Wollenw; *Pyrenochaeta lycopersici* Schn. et Gerlach; *Thielaviopsis basicola* (Berk et Br.) Ferraris, *Verticillium dahliae* Kleb.; and *Penicillium* sp.] and one antagonistic fungus (*Trichoderma viride* Pers. ex Fr.) were selected in the present study. The host plants from which the different fungi were isolated were: tobacco (*Nicotiana tabacum* L.) for *F. solani* and *T. basicola*, tomato (*Lycopersicon esculentum* L.) for *P. lycopersici* and *Penicillium* sp., and eggplant (*Solanum melongena* L.) for *V. dahliae*. The fungus *T. viride* was isolated from soil.

Sections of infected tissues were dipped in 1% sodium hypochlorite solution for 30 sec, washed three times with sterile distilled water, and blotted dry on sterile filter paper. The dissected samples were placed on a suitable medium in 9-cm Petri dishes. Potato dextrose agar (PDA), was used to isolate *F. solani*, *P. lycopersici*, and *V. dahliae*. For *F. solani* the Nash and Snyder PCNB medium

was also used. The carrot dish technique was employed to isolate *T. basicola*. Carrot discs (3–4 mm diameter) were soaked in H_2O_2 (20 vol) for 5 min, and placed in Petri plates containing sterile moist filter paper. Sections of tobacco roots infected with *T. basicola* were placed on carrot surfaces and incubated in the dark at 22–23°C. After the appearance of colonies, carrot discs were transferred to Petri plates containing PDA supplemented with streptomycin sulfate (100 mg/liter) and chloramphenicol (100 mg/liter). Stock cultures were maintained at 5°C on slants of PDA.

Fungal Bioassays. Bioassays were designed to evaluate: the fungitoxic effects of the rue extract, 8-MOP, 5-MOP, and 4-hydroxycoumarin. Appropriate amounts of lyophilized extract were mixed, before sterilization, with Czapek medium to obtain four final concentrations of 5, 10, 20, and 40 g/liter. In separate experiments, 8-MOP, 5-MOP, and 4-hydroxycoumarin were tested at four concentrations: 1/2, 1, 2, and 4 times those recovered in the rue extract (Aliotta et al., 1994). These active coumarins were evaluated only on three fungi: *P. lycopersici*, *F. solani*, and *T. viride*, chosen according to the sensitivity of response to the rue extract, which was, respectively, high, medium, and low. The solubility of 8-MOP, 5-MOP, and 4-OH coumarin was ensured by addition of a minute quantity of acetone in order to obtain a stock solution. Desired concentrations were prepared by diluting portions of the stock solution with Czapek medium. Additionally, a mixture of 5-MOP and 8-MOP was tested at four concentrations ranging from 64.8 to 129.6 mg/liter. In the presence of 4-OH coumarin, mycelial growth of the six fungi was not inhibited (data not shown), so this compound was not used in the mixture.

The agar was allowed to solidify in 9-cm Petri dishes, and experiments were initiated by placing 6-mm fungal plugs taken from the growing margins of 10-day-old cultures, mycelial side down, on the appropriate medium. Plates were incubated at 24–25°C. Radial growth for each test was recorded by taking the mean diameter of colonies from five plates. All treatments were replicated five times together with acetone-only and agar-only controls. The activity of each test compound was expressed as percent inhibition and, where appropriate, the log dose (concentration)–response relationship was linearized using probits and IC_{50}s (the concentration at which fungal growth was inhibited by 50% relative to controls) were determined (Finney, 1971). Data of percentage inhibition were analyzed by ANOVA test and means separation was made using Least Significant Difference (LSD).

RESULTS

Effects of Rue Lyophilized Extract. At the concentrations tested, lyophilized rue extract showed fungistatic activity against different fungal species (Figure 1).

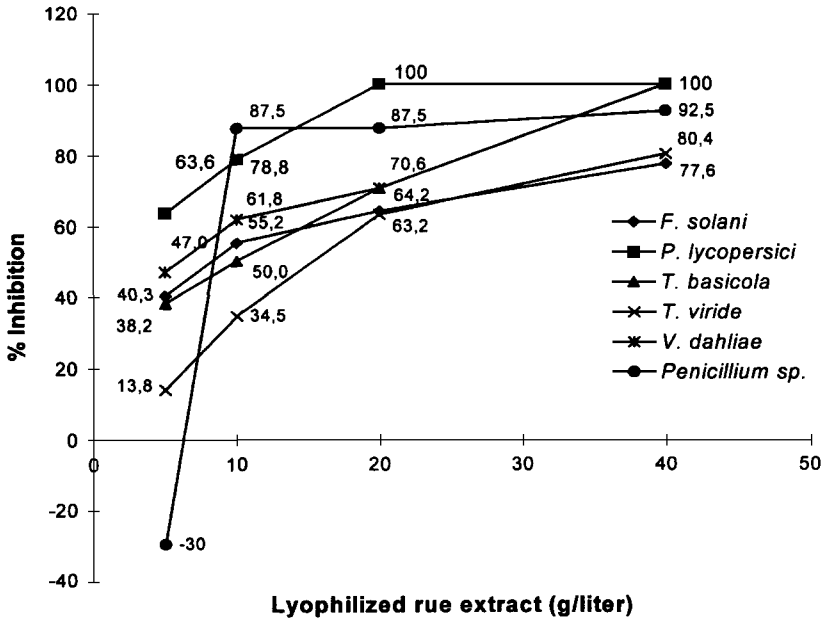

FIG. 1. Effect of increasing rue lyophilized extract concentrations (5–40 g/liter) on the radial growth rate of different fungal species. Each point represents the mean of 10 measurements. Standard errors were not presented on the graph because they do not exceed the size of the symbol. LSD > 4.7 for concentrations; >5.7 for fungi and >11.4 for the interaction concentration-fungus.

Except for *Penicillium* sp., significant reduction in the radial growth of all fungal species was observed (Figure 1). At the lowest concentration (5 g/liter), the response ranged from high inhibition for *P. lycopersici* (63.6%) and *V. dahliae* (47%), to medium for *T. basicola* (38.2%) and *F. solani* (40.3%) and low for *T. viride* (13.8%). *Penicillium* sp. had a dual response, exhibiting an increase of 30% radial growth at the lowest concentration, followed by a sharp inhibition (87.5%) at 10 g lyophilized extract/liter (Figure 1). Differences in the activity were explained by the calculated IC_{50} values ranging from 4.16 g/liter for *P. lycopersici*, which was the most susceptible, to 15.17 g/liter for *T. viride*. *Penicillium* sp. had an IC_{50} of 9.04 g/liter and the inhibition appeared when the concentration was identical to that of the crude extract.

Effects of 8-MOP, 5-MOP, and 4-Hydroxycoumarin. Unlike 8-MOP and 5-MOP, 4-OH coumarin did not influence the development of the fungi in any way. The effect of 8-MOP and 5-MOP was measured by IC_{50}; 8-MOP gave values of 39.9, 27.7, and 27.6 mg/liter, respectively, for *P. lycopersici*, *F. solani*, and *T.*

TABLE 1. REQUIRED CONCENTRATION TO INHIBIT GROWTH OF DIFFERENT FUNGAL SPECIES BY 50%

Fungal species	IC_{50} (mean ± SE)		
	Rue lyophilized extract (g/liter)	8-MOP (mg/liter)	5-MOP (mg/liter)
F. solani	8.25 ± 1.45	27.7 ± 3.5	6.77 ± 0.5
P. lycopersici	4.16 ± 0.07	39.9 ± 2.3	27.0 ± 0.3
T. basicola	8.43 ± 0.07		
T. viride	15.17 ± 0.04	27.6 ± 1.3	6.33 ± 0.1
T. dahliae	6.50 ± 0.10		
Penicillium sp.	9.04 ± 0.03		

viride; for 5-MOP, values were 27, 6.8, and 6.6 mg/liter (Table 1). Both 5-MOP and 8-MOP inhibited the radial growth of *F. solani* and *T. viride* more than *P. lycopersici* (Figure 2), while the rue extract was more active on *P. lycopersici.*

Effects of 8-MOP and 5-MOP Mixture. All concentrations tested completely inhibited the radial growth of all fungal species tested (data not shown). Mycelial growth of the six fungi was strongly inhibited in the presence of 64.8 mg (5-MOP + 8-MOP)/liter, the amount normally recovered in the rue extract.

Data of the percentage inhibition were normally distributed. Differences among concentrations and fungi were statistically significant by ANOVA tests. LSD values for rue extract and synthetic compounds are reported in Figures 1 and 2.

DISCUSSION

Earlier investigations by our group and others (Aliotta et al., 1992, 1993, 1995, 1996; Schopfer and Plachy, 1993) reported that an aqueous extract of *Ruta graveolens* L. significantly reduced radish and purslane germination and inhibited their radicle growth. Furthermore, 5-MOP was the most potent inhibitor of radish germination. In the present study, we determined that the same compounds within the lyophilized rue extract may be considered as fungistatic against the tested pathogenic fungi.

Rue extract showed significant activity against the fungal species tested, indicating its antifungal potential (Wilson et al., 1997). In the agar dilution assay, fungal hyphal growth was not completely inhibited, showing that the effects of rue extracts were fungistatic rather than fungicidal. Interestingly, the radial growth of *Penicillium* sp., was promoted when grown in the presence of the lowest concentration (5 g/liter) of the rue lyophilized extract. Species specificity and the concentration-dependent action of allelochemicals have been widely

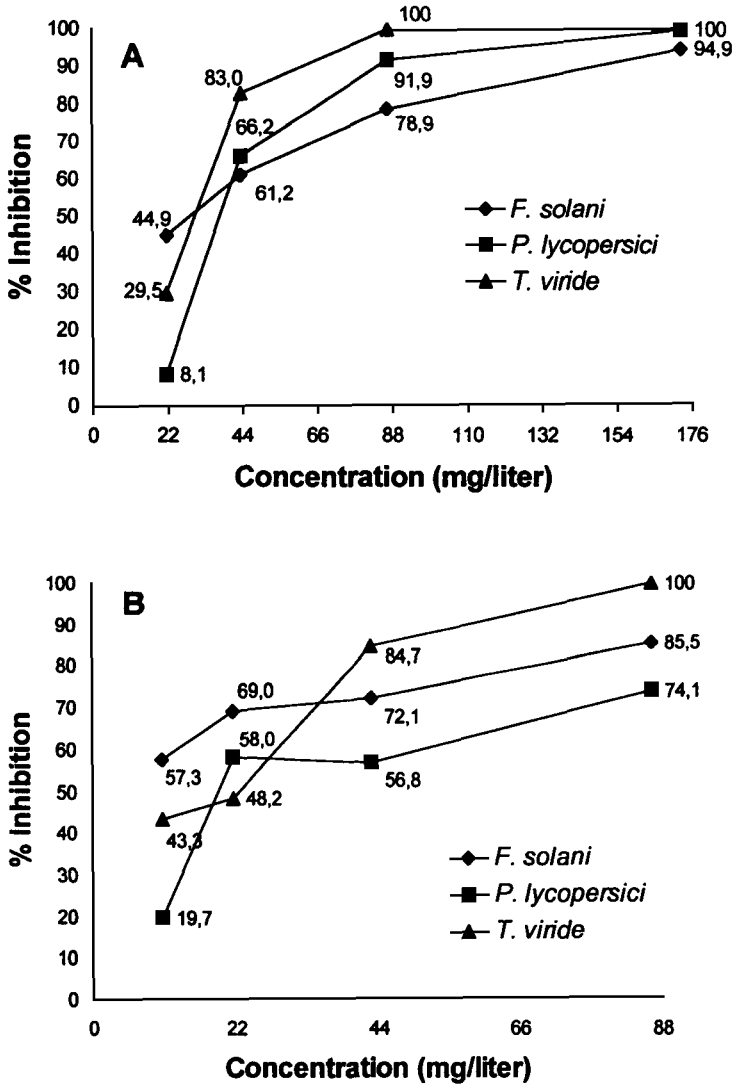

FIG. 2. Effect of 8-MOP (A) and 5-MOP (B) at four different concentrations on the mycelial growth rate of *F. solani*, *P. lycopersici*, and *T. viride*. Each point represents the mean of 20 measurements. Standard errors were not presented on the graph because they do not exceed the size of the symbol. LSD > 6.9 for concentrations; >6.0 for fungi and >11.9 for the interaction (8-MOP). LSD > 3.7 for concentrations; >3.3 for fungi and >6.5 for the interaction.

observed (Rice, 1984, 1995). This behavior plays an important regulatory role in nature by determining the interactions among the organisms with different susceptibilities (Rice, 1984).

None of the concentrations of 4-hydroxycoumarin tested was found to inhibit the growth of any of the fungal species, although coumarins were reported to have significant biological activity (Zobel and Louis, 1990; Zobel and Brown, 1995). Results obtained from all in vitro assays against various fungal species indicate 5-MOP to be the more active (IC_{50} value 6.3 mg/liter on *T. viride*). Compared to the rue extract, the mixture of the synthetic compounds 8-MOP and 5-MOP, at the concentrations at which they naturally occur in the crude rue extract, was found to be more inhibitory to growth of different fungal species. This feature could be due to the complexity of the interactions among the constituents of the extract and to the *status* of the active compounds interacting with light (Edelson, 1986; Downum et al., 1989; Aliotta et al., 1992).

All but one of the fungi tested were plant pathogens, and pure compounds were most effective against the antagonistic fungus *T. viride*, rather than the disease agents. On the other hand, the rue extract was more active against the pathogens. Intuitively, it would seem that the combined application of rue extract and conidia of *T. viride* (Agrios, 1988) to the soil, could be the basis of a useful method to control plant disease.

Much research remains to be done to ascertain the potential of this simple rue infusion as a cost-effective tool for pest management in crops (e.g., its biodegradability and toxicity).

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CHEMICAL CHARACTERIZATION OF URINARY PHEROMONES IN BROWN ANTECHINUS, *Antechinus stuartii*

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Abstract—In the small dasyurid marsupial, *Antechinus stuartii*, males exhibit scent-marking in the form of cloacal marking of nesting areas during the breeding season. Females of this species show no such behavior. To characterize the potential male pheromonal scent signal, urine-derived volatiles from sexually active males were analyzed by GC-MS and compared to that of females and a castrated male. More than 10 urinary compounds were identified. A series of homologous methylketones was observed in both males and females, whereas aldehydes were present only in female urine. Urine from the castrate was virtually compound-free except for minute concentrations of a compound tentatively identified as 2,4-dithiapentane. This compound was also found in one of the sexually active males. The GC profiles of the sexually active males contained high concentrations of two pyrazine derivatives and four methylketones that were not detected in the profiles of either females or the castrate. These compounds may influence social communication in the brown antechinus, *Antechinus stuartii*.

Key Words—Marsupial, pheromone, social communication, pyrazines.

INTRODUCTION

Scent-marking and subsequent olfactory investigation of chemical signals are fundamental components of social interactions within the majority of mammalian

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species. Frequently, however, the function of a specific mammalian social odor cannot be accurately determined; odors often convey various information such as about sex, reproductive or emotional state, and even individual identity (Brahmachary, 1986).

In mammals, scent-marking is accomplished by the use of urine (Stralendorff, 1987), feces, and specialized glands, such as the sternal gland in the marsupial sugar glider (Stoddart et al., 1994), the temporal gland of elephants, and supraorbital and interdigital glands of many ungulates (Brahmachary, 1986).

Research into the chemistry of mammalian pheromones, which builds on the success of chemical investigations of insect pheromones, has mainly focused on social conditions that affect sexual maturation and reproduction in rodents (Drickamer, 1984; Jemiolo et al., 1994).

Within the metatheria, little is known about the chemistry of chemosignals, although their involvement in reproduction and social organization is well documented (Fadem et al., 1989). Woolhouse et al. (1994) have described a possible paracloacal and sternal gland pheromone in the brushtail possum *Trichosorus vulpecula* that may act as an intraspecies attractant. Furthermore, partially racemic compounds have been identified as possum urinary metabolites (Carman and Klika, 1992), the function of which, however, is unknown.

The brown antechinus, *Antechinus stuartii* (Marsupialia: Dasyuridae), a small dasyurid marsupial that inhabits the rain forest of the eastern coast of Australia, is thought to use chemical signals for social interactions. The brown antechinus is a mainly solitary species; however at the start of the breeding period, individuals may form groups of up to two dozen animals (Cockburn and Lazenby-Cohen, 1992).

In *A. stuartii*, males possess both a sternal gland and a cluster of paracloacal glands that are thought to produce chemical signals for intraspecific communication (Toftegaard and Bradley, submitted). Females do not possess these glands, but synchrony of female ovulation has been reported and is thought to be pheromonally mediated. Possible sources may be urine or feces (Scott, 1986).

Although sternal marking has not been observed, cloacal marking, where the animal propels itself forward while pressing its cloaca to the substrate, has been reported in males during the breeding season (Braithwaite, 1974). It is believed that cloacal marking in this species functions as a threat to other males and an aggregation stimulus to females, the implication being that the urine of male *A. stuartii* contains a characteristic, possibly androgen-dependent chemical signal that differs from that of the female and whose volatile component is capable of communicating chemical information over extended distances.

Although evidence from studies of several mammal species suggests that nonvolatile components are critical and the vomeronasal organ plays a key role in perceiving these signals, this study of urinary volatiles represents our first step

in identifying the proposed sexually dimorphic components of *A. stuartii* urinary chemosignals. Analysis of urinary nonvolatiles will be reported elsewhere.

METHODS AND MATERIALS

A total of 11 adult *A. stuartii* were used (seven males, three females, and one castrated male) as urine donors. The animals were housed individually in cages, and the colony room was kept at 22–24°C with a 16L : 8D cycle. Cat food (Wiskas and Wiskettes dry food, supplied by Uncle Ben's of Australia) and water were available ad libitum.

Urinary samples used for chemical analyses were obtained by placing the donor in an apparatus modified from Purvis and Haynes (1978) overnight (from 10 : 00 AM to 10 : 00 AM). Samples were collected in clean glass vials, stored at −20°C, and analyzed within one month.

Solid-phase microextraction (SPME) (Zhang and Pawliszyn, 1993; Coleman, 1996) utilizing a 100-μm poly(dimethylsiloxane) (PDMS) -coated fiber (Supelco, Bellefonte, Pennsylvania) was used to sample the headspace of urine samples. Then 0.5-ml aliquots of urine were diluted with an equal volume of saturated sodium chloride solution in a 4-ml headspace vial. The fiber of the SPME device (Supelco) was exposed to the headspace of the stirred solution for 20 min, then withdrawn, immediately inserted into the GC injector, and exposed as data acquisition was begun. The fiber was left in the injector for 10 min. GC-MS analyses were performed on a Trio-2000 instrument (Fisons Instruments, VG Biotech, Altringham, UK). The capillary column (0.25 μm, 0.25 mm ID, 30 DB-5 ms, J&W Scientific, Folsom, California) was held at 40°C for 2 min, then raised to 260°C at 10°C/min, and held at this temperature for 6 min. Injection was splitless for 1 min with the injector temperature 250°C. The additional time of heating of the fiber ensured complete reconditioning. Mass spectra were generated by 70 eV electron impact.

RESULTS

The gas chromatograms displayed in Figure 1 show the volatile profiles obtained from sexually intact male urine (Figure 1, profiles 1 and 2), castrate male urine (Figure 1, profile 3), and female urine (Figure 1, profile 4). Fourteen volatile compounds were identified by best-fit comparisons of the mass spectra and retention time using computerized databases. Identified compounds included pyrazines (Figure 2), methylketones, and aldehydes (Table 1).

Through careful inspection of the GC-MS profiles and analysis of peak areas of urinary volatiles obtained from the three experimental groups (males, females, and castrate), it was established that nine constituents showed signifi-

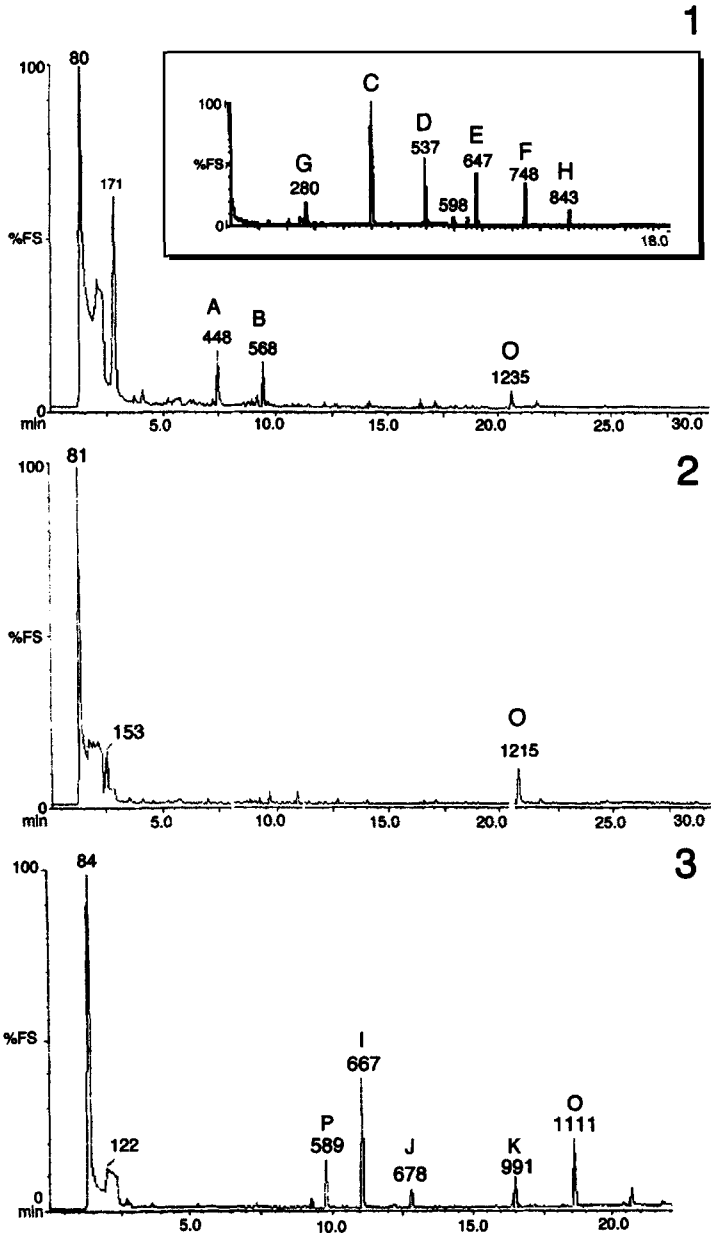

FIG. 1. GC-MS chromatographic profiles of the volatile compounds in urine from sexually intact male (1 and 2), castrated male (3), and female (4) of *A. stuartii*.

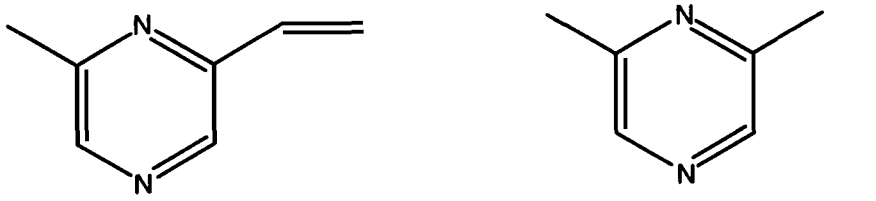

FIG. 2. Structural composition of pheromonal pyrazines present in *A. stuartii* urine.

cant differences in their chromatographic peak area (concentrations) according to reproductive status and gender of an animal (Table 2).

All three types of urine were characterized by the presence of one or a few volatiles in high concentration. All contained *N*-butyl benzene sulfonamide (peak O), which is believed to be a contaminant introduced during the prepara-

TABLE 1. CLASS AND STRUCTURE OF VOLATILE COMPOUNDS IDENTIFIED IN URINE OF MALE AND FEMALE *A. stuartii* BY GC-MS ANALYSIS

Class and structure of urinary volatiles	Peak code	Molecular weight (Da)	Elemental formula
Pyrazines			
2,6-Dimethyl pyrazine	A	108.14	$C_6H_8N_2$
2-Ethenyl-6-methyl pyrazine	B	120.15	$C_7H_8N_2$
Ketones			
2-Heptanone	C	114.19	$C_7H_{14}O$
2-Octanone	D	128.22	$C_8H_{16}O$
2-Nonanone	E	142.24	$C_9H_{18}O$
2-Decanone	F	156.27	$C_{10}H_{20}O$
2-Hexanone	G	100.16	$C_6H_{12}O$
2-Undecanone	H	170.29	$C_{11}H_{22}O$
Aldehydes			
Nonanal	I	142.24	$C_9H_{18}O$
Decanal	J	156.27	$C_{10}H_{20}O$
Undecanal	K	170.30	$C_{11}H_{22}O$
Miscellaneous			
Benzaldehyde	L	106.13	C_7H_6O
Decanol	M	158.29	$C_{10}H_{22}O$
2,4-Dithiapentane	N	108.23	$C_3H_8S_2$
N-Butyl benzene sulfonamide	O	213.30	$C_{10}H_{15}NO_2S$
Limonene	P	136.24	$C_{10}H_{16}$

TABLE 2. VOLATILE COMPOUNDS IDENTIFIED IN *A. Stuartii* URINE ACCORDING TO GENDER AND REPRODUCTIVE STATUS

Class/peak code	Males	Females	Castrate
A	×		
B	×		
C	×	×	
D	×		
E	×		
F	×		
G	×		
H			
I		×	
J		×	
K		×	
L			
M			
N	×		×
O	×	×	×
P	×	×	

tion of samples for GC-MS analysis. Urine from intact males contained a series of methylketones (peaks C–H) such as 2-heptanone, 2-hexanone, 2-octanone, 2-nonanone, 2-decanone, and 2-undecanone. Only 2-heptanone (peak C) was present in female urine.

Only urine from sexually intact males contained 2,6-dimethylpyrazine (peak A) and 2-ethenyl-6-methyl pyrazine (peak B). In contrast to the male profiles, urine from females contained a small number of aldehydes (peaks I–K) such as decanal, nonanal, and undecanal. Urine from the castrated male contained *N*-butyl benzene sulfonamide and only trace amounts of 2,4-dithiapentane, a compound also found in one sexually intact male. Compounds such as benzaldehyde, decanol, and limonene were also identified in some individuals. However, since these compounds failed to show consistency in all animals examined, their presence was only noted and not identified in Figure 1.

DISCUSSION

The SPME method used in this study was chosen to identify major volatile components present in the urine of *A. stuartii*. Chemical analysis of *A. stuartii* urine revealed the presence of 14 volatile compounds. This means there are relatively few constituents in *A. stuartii* urine in comparison to the volatile-rich urine of wolves and rodents, which may contained over 100 compounds (Schwende et al., 1986).

2-Heptanone and benzaldehyde identified in male *A. stuartii* urine also occur in male wolf (*Canis lupus*) urine at the time of sexual maturation (Raymer et al., 1986), and in the urine of house mice (*Mus musculus*) and pine voles (*Microtus pinetorum*) (Boyer et al., 1989). Furthermore, 2-heptanone has been identified as a honeybee pheromone (Lensky and Cassier, 1995) and as an attractant in wasps (Weston et al., 1997).

The observed apparent sexual dimorphism in urine constituents in *A. stuartii* is of particular interest with regard to the presence of pyrazine derivatives. Such derivatives have also been noted in tree shrews (*Tupaia belangeri*) (Stralendorff, 1987), where male-specific 2,5-dimethylpyrazine was identified. The presence of this compound was interpreted to be directly related to the activation of male chinning behavior.

In mice, male urinary cues may delay sexual maturation in female conspecifics (Colby and Vandenbergh, 1974). The identification of urinary pyrazines as key regulatory components in reproduction has shown that long-term exposure of female mice to 2,5-dimethylpyrazine may inhibit their overall reproductive fitness (Jemiolo and Novotny, 1993).

Novotny et al. (1986) report that 2,5-dimethylpyrazine is present in the adrenal gland of mice. Further studies have shown that adrenalectomy, but not ovariectomy, eliminates the biological activity of excreted urine in causing a delay in sexual maturation of juvenile mice (Drickamer and McIntosh, 1980). Furthermore it has been proposed that tetraethylpyrazine and 2,5-dimethylpyrazine may modulate the gonadotropin-releasing hormone and/or gonadotropic hormone release from the hypothalamic–pituitary axis in juvenile female rats (Yamada et al., 1989).

Pyrazines have been identified as volatile constituents in various foods (Buttery et al., 1971). They are formed naturally during the roasting process from carbohydrates and amino acids by a Maillard reaction (Rizzi, 1972), and could possibly be artificially produced from other urine components during heating (e.g., in the gas chromatograph injector). Evidence that they are not is provided by the observed dichotomy in identically treated samples, and furthermore, their precursors in the roasting process are nonvolatile, and thus they would not transfer to the urine headspace.

Pyrazines are naturally produced by the metabolic activity of various bacteria. Therefore, regardless of whether a possible chemosignal substance is derived from the pyrazine compounds or their metabolites, they do appear to be male-specific in *A. stuartii*.

Many mammals can discriminate between scent marks produced by conspecifics of different sexes and different physiological states. Within individuals, this discrimination could be based on singular characteristic scent components or on a particular, finely tuned mixture of components.

In *A. stuartii*, the sexual dimorphism in urine volatile components could

imply either a single functional activator component, such as a pyrazine, or a particular mixture of certain components. We have data strongly suggesting that urine from both male and female conspecifics may affect both the pituitary–adrenocortical and reproductive status in conspecific males (Toftegaard and Bradley in preparation). However, it is not yet known whether the signal may be androgen-dependent or if it is even possible to identify and isolate one pheromonal component as a singular activator.

The focus of this study has been the chemical characterization of the volatile compounds of *A. stuartii* urine. To address which component(s) of the volatile fraction may influence social and reproductive behavior, a bioassay testing conspecific responsiveness to the various observed volatiles has been completed, the results of which will be reported elsewhere. Further experiments are underway in our laboratory to examine the possibility of androgen dependency in urinary volatile constituents. Moreover, experiments using more sensitive purge-and-trap methods are planned to fully characterize the volatile components of *A. stuartii* urine.

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DIFFERENTIAL SENSITIVITY OF MOSQUITO TAXA TO VEGETABLE TANNINS

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Abstract—The sensitivity of larval Culicidae to vegetable tannins was investigated in different taxa representative of the fauna from alpine hydrosystems (*Aedes rusticus*, *Culex pipiens*) and foreign noxious fauna (*Aedes aegypti*, *A. albopictus*). Bioassays reveal that tannic acid at concentrations of 0.1–6 mM is significantly more toxic for *C. pipiens* than for *Aedes* taxa, and *A. aegypti* is more sensitive than *A. albopictus* and *A. rusticus*. Comparison of the rank order of sensitivity among taxa with the associated levels of cytochrome P-450, esterase, and glutathione-*S*-transferase activities suggests that cytochrome P-450 and esterases may be involved in the detoxification of tannins. A possible involvement of these detoxifying enzymes is also revealed in vivo by the synergistic effects of *S,S,S*-tributyl phosphorotrithioate (esterase inhibitor) and piperonyl butoxide (P-450 inhibitor). The differential sensitivity to tannins among taxa is discussed in terms of ecological implications within mosquito communities from alpine hydrosystems, where the acquisition of tannins–detoxifying enzymatic systems may be considered as a key innovation.

Key Words—Tannic acid, larvicidal activity, *Aedes rusticus*, *Aedes albopictus*, *Aedes aegypti*, *Culex pipiens*, bioassays, enzyme assays, cytochrome P-450, glutathione-*S*-transferases, esterases.

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INTRODUCTION

Larval biological control is generally a more effective method of reducing harmful populations of Culicidae than spraying adults with residual insecticides that may be not safe for the environment and that may induce resistance (Tellier et al., 1991). In this respect, ecological mapping of vegetation zones is proving useful in larval surveys (Gabinaud, 1987), and numerous studies on the association between vegetation and mosquito breeding have identified various types of plants or plant communities characteristic of certain culicine species (Provost, 1975; Rioux et al., 1968). Habitat water contains both nutrients and deterrents for mosquito larvae, but the significance of intrinsic chemical properties of water for habitat segregation or interspecific interactions among taxa is poorly understood, despite a few analyses on effects of water conditions on the distribution of some particular species (Sota, 1993).

Biogeographic studies on the Culicidae from areas that may periodically flood in the Rhône-Alpes region of France have indicated a clear correlation between alder [*Alnus glutinosa* (L.) Gaertn.] communities and larval habitats of several *Aedes*, *Culiseta*, and *Anopheles* species, whereas *Culex pipiens* L. is absent from these hydrosystems (Pautou et al., 1973). Preliminary ecophysiological investigations have led to the suggestion that habitat segregation may be due to alder leaves falling and decomposing in the water in *Aedes* spp. breeding sites. The tannins and other phenolic compounds thus released may be responsible for qualitative discrimination among larval populations (Rey et al., 1996). Tannins are among the more abundant water-soluble, secondary plant compounds (Harborne, 1982) and may bind with proteins (reviewed in De Veau and Schultz, 1992). Among their various ecological effects (reviewed in Appel, 1993), they may be quite harmful to insects (reviewed in Ayres et al., 1997). Their adverse effects on the survival of phytophagous Lepidoptera larvae have been extensively studied (Barbehenn and Martin, 1994), but data on larvae of Diptera are available only for a few *Aedes* taxa (Sota, 1993; Mercer and Anderson, 1994). Differential sensitivity to tannins is emphasized in this study of two *Aedes* and *Culex* species representative of the alpine target fauna and two foreign harmful *Aedes* species exploiting a wide variety of habitats. A comparison of their larval performances was conducted in water containing different concentrations of tannic acid to evaluate tannins as biological control agents. This is the first aim of our work.

Enzymatic detoxification is a primary adaptation of insects to plant allelochemicals (Ahmad et al., 1986). In this respect, insect differential sensitivity to those substances has been correlated with their ability to metabolize them through various detoxifying enzyme systems, including cytochrome P-450 monooxygenases (P-450) (Brattsten, 1992), glutathione-*S*-transferases (GST) (Wadleigh and Yu, 1988), and esterases (Smirle et al., 1996). Consequently, a

comparative analysis of these enzymatic activities among currently studied *Aedes* and *Culex* species may help us to better understand their own sensitivity to tannins and to further elucidate the mechanistic basis of the differential tolerance of insects to phenolics, which is still poorly understood (Appel, 1993). This is the second purpose of this work.

METHODS AND MATERIALS

Insects. In all taxa, fourth instars were analyzed. Among the alpine taxa, field strains originated from a resurgence (*Aedes rusticus* Rossi) and a lagooning basin (*Culex pipiens* L.). Larvae were held in the laboratory several days before use. Foreign species were obtained from colonies raised in the laboratory (*A. albopictus* Skuse: Italian strain; *A. aegypti* L.: Bora-Bora strain).

Chemicals. Chemicals used in bioassays were tannic acid (MW = 1700 g/mol) from Prolabo; piperonyl butoxide (PB) from Calbiochem; and *S,S,S*-tributyl phosphorotrithioate (DEF) from Interchim. Chemicals used for microsomal preparations were dithiothreitol (DTT) and ethylenediamine tetraacetic acid (EDTA) from Boehringer and phenylmethylsulfonyl fluoride (PMSF) and *N*-ethylmaleimide from Sigma. Protein assays were conducted with bovine serum albumin (BSA) from Boehringer. Enzyme assays were performed with α- and β-naphthylacetate (α-, β-NA); reduced glutathione; 7-ethoxycoumarine; nicotinamide adenine dinucleotide phosphate, reduced form (NADPH); 2α-, 2β-, 6α-, 6β-, 15α-, and 16α-hydroxytestosterone (2α-OH, 2β-OH, 6α-OH, 6β-OH, 15α-OH, and 16α-OH) from Sigma; 1-chloro-2,4-dinitrobenzene (CDNB) from Aldrich; and [^{14}C]testosterone (1.85 Gbq/mmol) from Amersham. TLC silica gel plates (0.25 mm thick) were from Merck-Clevenot.

Bioassays. Resistance characteristics of the different taxa were analyzed with bioassays conducted in triplicate, at 20°C, on homogeneous samples (N = 25), in 50-ml solutions. Controls were reared in tap water, and treated specimens were reared in tannic acid solutions varying in concentration from 0.1 to 6 mM (Figure 1 below), with pH adjusted to 8.5. The mortality rate was checked 24, 48, and 72 hr after beginning of treatment. After correction according to Abbott's formula (Abbott, 1925), mortality data were analyzed with a log-probit program (Raymond, 1985).

The action of DEF as an esterase inhibitor (Yuan and Chambers, 1996) and PB as a P-450 inhibitor (Motayama et al., 1991) was investigated by exposing *A. albopictus* and *A. aegypti* larvae to a standard dose of synergist that had no effect on the viability of controls (1.1 ppm for DEF and 4 ppm for PB) 4 hr before tannic acid treatment.

Enzyme Assays. Enzyme activities were analyzed among the different taxa with the microsomes (P-450) and the supernatant (GST, esterases) of homog-

enates from whole-body extracts as the enzyme sources. For microsomal preparation, specimens were homogenized on ice in 50 mM phosphate buffer, pH 7.2, containing 0.4 mM PMSF, 1 mM EDTA, 0.1 mM DTT, and 0.5 mM *N*-ethylmaleimide, then centrifugated at 10,000*g* for 15 min at 4°C. The resulting supernatant was centrifugated at 100,000*g* for 60 min at 4°C, and the microsomal pellet was resuspended in the same buffer with 20% glycerol (v/v). Protein content was determined by the method of Bradford (1976), with BSA as the standard.

P-450 activities were determined quantitatively, through measurement of the ethoxycoumarin-*o*-deethylase (ECOD) activity, according to Ulrich and Weber (1972), and qualitatively, through study of the hydroxylation of [^{14}C] testosterone by microsomes, according to Larroque et al. (1988), modified by Cuany et al. (1990), on TLC silica gel plates.

GST activities were determined on 250 μg proteins in 0.05 M phosphate buffer containing reduced glutathione at 0.1 and 1 mM and CDNB at 1, 2, 5, 10, and 20 mM. The variations in optical density were recorded for 1 min at 340 nm at 25°C (Habig et al., 1974). Kinetic parameters (K_m = Michaelis constant, and V_{max} = maximum velocity) were determined according to Lineweaver and Burk (1934).

Esterase activities were assayed on 200 μg of protein by the method of Van Asperen (1962), by using 1-NA and 2-NA as substrates. The concentrations of α- and β-naphthol produced by hydrolysis of 1-NA and 2-NA were determined by a standard curve.

RESULTS

Larval Resistance Characteristics of Different Taxa. Tannic acid treatment caused linear mortality ($P > 0.1$) in all dose–response relationships (for example, after 24 hr of treatment; Figure 1), indicating homogeneity of the resistance/sensitivity within the four taxa. However, striking differences were observed among taxa, with *C. pipiens* appearing highly sensitive to tannic acid treatment in comparison to the three *Aedes* species. Among *Aedes* taxa, *A. aegypti* appeared more sensitive than *A. albopictus*, which was more sensitive than *A. rusticus*.

Previous addition of PB or DEF in bioassays caused a significant decrease of larval resistance to tannic acid among the taxa, as exemplified in Figure 2 for *A. aegypti*. This synergistic effect globally appeared more potent for PB than for DEF.

Enzymes Activities. Comparison of P-450 activities revealed several differences among taxa. Quantitative differences were observed concerning the overall P-450 activities through the ECOD method (Table 1). Comparison with bioassay data (Figure 1) indicates that such differences may be correlated with variations in the degree of resistance to tannins among taxa, as well as between *Culex* and

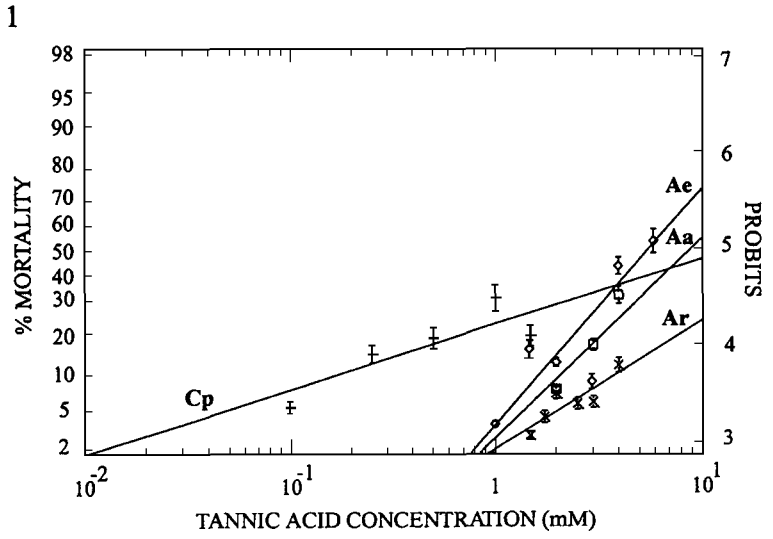

FIG. 1. Differential sensitivity to tannic acid among *Culex pipiens* (Cp), *Aedes aegypti* (Ae), *A. albopictus* (Aa), and *A. rusticus* (Ar) larvae: dose–mortality curves after 24 hr of treatment.

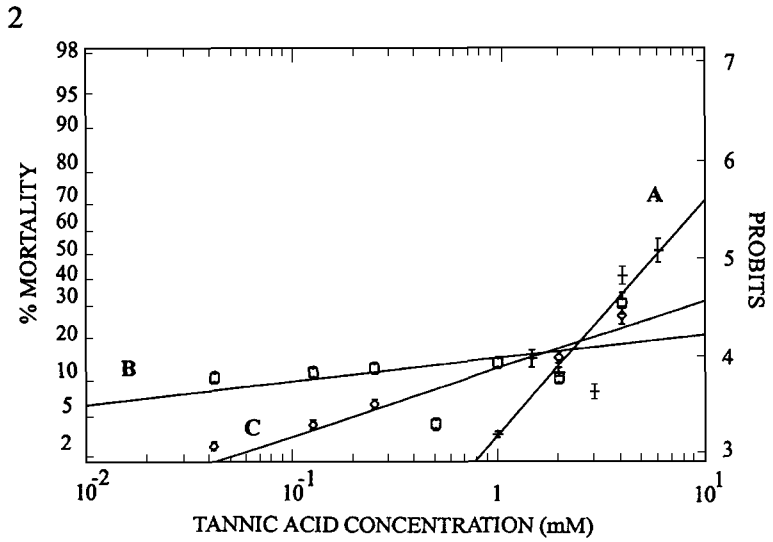

FIG. 2. Synergistic effects of DEF and PB against *Aedes aegypti* larvae: comparison of dose/mortality curves after 24 hr of tannic acid treatment in absence of synergists (A), in presence of PB (B), and in presence of DEF (C).

TABLE 1. COMPARISON OF SPECIFIC DETOXIFYING ENZYME ACTIVITIES IN *Aedes albopictus* (Aa), *A. aegypti* (Ae), *A. rusticus* (Ar), AND *Culex pipiens* (Cp) RELATIVE TO THEIR SENSITIVITY TO TANNIC ACID[a]

Taxa	P-450 (ng 7-hydroxycoumarin/ mg protein/ min)	Esterases		GST	
		a (ng 1-naphthylacetate/ mg protein/min)	b (ng 2-naphthylacetate/ mg protein/min)	V_{max} (mg protein/ min)	K_m (mM)
Ar	1.37	1475.1	16572.7	223	953
Aa	1.06	1414.1	16231.2	740	107
Ae	0.79	510.8	15035.9	100	593
↓ Cp	0.62	285.9	7009.9	242	412

[a]Vertical arrow indicates the increasing sensitivity among taxa.

Aedes genera, and among *Aedes* species. Qualitative variations were observed on autoradiograms of TLC plates after incubation of ^{14}C-labeled testosterone with microsomes from the different *Aedes* taxa (Figure 3). Androstenedione and acid metabolites do not appear to be P-450 dependent, because they were formed even in the absence of NADPH (Figure 3: standard), but 20α-, 2α-, 16β-, 2β-, 6β-, 7α-, 15β-, and 16α-OH were NADPH dependent, and CO sensitive (not shown), indicating their P-450 dependence. Among those metabolites, the 15β- and 16β-OH appeared characteristic of *A. albopictus*.

In the same way, significant differences in esterases activities appeared among taxa, particularly with 1-NA (Table 1), which are congruent with the level of resistance to tannic acid in each taxa (see Figure 1). However, comparative analysis of GST activities among taxa (Table 1) showed no obvious degree of correlation with their levels of resistance to tannins (Figure 1).

DISCUSSION

Larvae from mosquito taxa representative of alpine communities (*A. rusticus*, *C. pipiens*) and two foreign species (*A. albopictus*, *A. aegypti*) show different sensitivities to tannins. Similar data were reported for some other Culicidae taxa (reviewed in Mercer and Anderson, 1994) and phytophagous insects, especially lepidopteran caterpillars (Berenbaum, 1983) and Orthoptera (Bernays et al., 1981). Such a variable degree of tolerance to tannins among taxa is correlated with different levels of P-450 and esterase activities, suggesting that those enzymatic systems are implicated in the detoxification of these xenobiotics. Enzyme assays coupled with biological assays are potential tools for estimating the resistance of field strains to xenobiotics (reviewed in Harold and Ottea, 1997).

Ecological Significance. The differential larvicidal activity of tannic acid

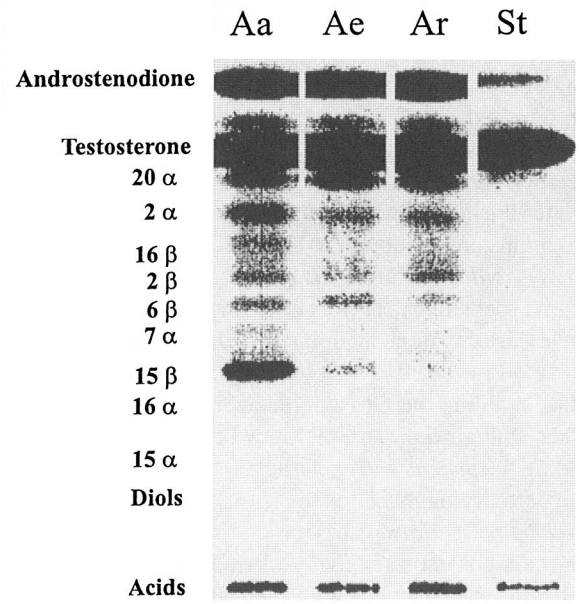

FIG. 3. Autoradiogram of metabolites after in vitro metabolism of [^{14}C]testosterone by microsomes from *Aedes albopictus* (Aa), *A. aegypti* (Ae), and *A. rusticus* (Ar) larvae. St = standard.

against Culicidae taxa revealed by the current laboratory bioassays corroborates previous field observations showing strict correlation between alder communities and larval habitat of specific mosquito species (Pautou, 1981). Together the results indicate the role of plant tannins, and, more generally, of natural phenolic compounds for habitat selection in mosquito communities from alpine hydrosystems, as has been observed for some other Culicidae taxa from biotopes rich in environmental tannins (Sota, 1993).

This vegetable tannins–mosquito interaction is not surprising since several hydrophytes are known to produce and/or release chemical substances that are antagonistic to the development of aquatic invertebrates (Dhillon et al., 1982), including mosquito larvae (Paradise and Dunson, 1997). Moreover, tannins are among the most biologically active solutes released in the aquatic environment by decaying vegetation (Swain, 1979). Such plant–insect interaction may come from both herbivory and allelopathy because tannins from the leaf litter may constitute both nutrients and deterrents in habitat water for Culicidae larvae. The ecological significance is of interest because of the highly selective environmental conditions created in such biotopes and the biological importance of hydrophyte communities (e.g., *Alnus* spp., *Populus* spp., *Quercus* spp.: Pautou et al., 1973) producing

high levels of foliar water-soluble tannins. Primary production of tannins in forests has been estimated at up to 800 g/m^2/yr (Ayres et al., 1997). Because tannins may resist microbial degradation (Grant, 1976), they can be concentrated by evaporation of water in poorly drained areas to reach levels comparable to those used in our laboratory bioassays. Culicidae larvae that are able to survive under tannin-producing vegetation have to remain in contact with tannins released from the leaf litter into the water during the whole larval period, which is relatively long. The toxic effects of tannins on mosquito larvae appear more acute than those of bioinsecticides, such as *Bacillus thuringiensis* Berliner, which causes fatal lesions in the midgut epithelium of sensitive taxa (Rey et al., 1999). Thus, the ability to survive in water with high levels of tannins in alpine hydrosystems appears as a key innovation (Berenbaum et al., 1996) that may have promoted diversification in breeding sites of Culicidae, enabling them to colonize zones corresponding to a large geographic area. This ecological opportunity may be possible for a limited number of taxa (e.g., including *A. rusticus* and excluding *C. pipiens*), and it implies the acquisition of specific detoxifying mechanisms.

Toxicological Implications. Enzyme-dependant survival in a chemically refractory medium is common for many plant–insect interactions as specialization in insects has been associated with their ability to metabolize plant toxins (Berenbaum et al., 1996) through several possible ways. Tannins are metabolizable in the insect midgut by P-450s (Ahmad et al., 1986). P-450-associated enzymes are heme-bound, differentially inducible proteins reported in many organisms to play a major role in the detoxification of a wide array of xenobiotics (Brattsten, 1992; Frank and Fogleman, 1992), including many secondary plant compounds (Yu, 1987). Such a polyvalency in the oxidative metabolism is due to the large number of families or forms of P-450 that are expressed in each organism.

Larval survival on tannin substrate was reduced in insensitive taxa by addition of PB, revealing a synergistic effect of this typical P-450 inhibitor (Motayama et al., 1991). Similar results were reported in phenolic glycoside detoxification by Lepidoptera larvae (Berenbaum, 1991). However, incomplete reduction of mosquito larval tolerance by PB suggests that additional mechanisms are implicated in tannins detoxification. There is a positive correlation between the P-450 activity levels and the levels of resistance among the taxa studied. However, the total in vitro P-450 activity in each taxa may have been underestimated, because it was measured in microsomes prepared from undecapitated specimens. Measurable P-450 activity was consistently found to be 2.0- to 3.7-fold greater in insects when assayed using decapitated versus intact specimens, indicating a biasing effect of endogenous head inhibitors such as eye pigments (Danielson et al., 1994). Moreover, because of the large number of P-450 isozymes that may be implicated in detoxifying systems (Nelson et al., 1993), the comparison of microsomal metabolization of testosterone may be an indicator of the degree of isozyme multiplicity involved in the resistance against tannins. As in the case of *Drosophila* (Cuany

et al., 1990), Culicidae microsomes gave numerous oxidative metabolites, some of them appearing characteristic of some taxa. This appears to be the case for the 15β- and 16β-hydroxytestosterone in *A. albopictus*. The 15β-hydroxytestosterone is known to be produced in man by the CYP 3A4 isozyme (Guengerich et al., 1986), and the 16β-hydroxytestosterone is produced by the CYP 2B1 isozyme (Waxman et al., 1985). Thus, if each of these hydroxylations is performed by a specific isozyme, as in mammalian systems (Waxman et al., 1983; Larroque et al., 1988), the different pathways of testosterone metabolism currently observed among the *Aedes* taxa suggest that different P-450 forms may be involved in the detoxification of tannins by *Aedes* larvae. Such an involvement of diverse P-450s has been observed in other Culicidae taxa (Scott et al., 1994), and immunochemically in Lepidoptera larvae (Cohen et al., 1990).

The role of esterases in the metabolization of tannins is not surprising because there is an abundance of esters groups within hydrolyzable tannins (Towers et al., 1964). Esterases play an important role in resistance to some insecticides (Karoly et al., 1996) and to vegetable phenolic glycosides (Beninger and Abou-Zaid, 1997). Differences in 1-NA esterases activity were clearly positively correlated with resistance to tannins among all Culicidae taxa studied. Berenbaum (1991) has suggested that esterases may function as the primary detoxification enzyme system while P-450 may neutralize those toxic molecules that escape esterase action.

Glutathione-*S*-transferases probably do not play a significant role in the resistance of Culicidae larvae to tannins, in contrast to that reported for the detoxification of numerous insecticides (Clark, 1990) and plant allelochemicals (Lee, 1991; Harshman et al., 1991; Leszczynski et al., 1994), by insects including mosquitoes (Mourya et al., 1993).

Vegetable tannins and, more generally, phenolic compounds appear to be strongly involved in the mosquito–vegetation interaction in alpine hydrosystems. A better knowledge of such chemical messengers will allow us to use them as possible phytoinsecticides to improve our ability to make sound management decisions in the future.

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SHIELD DEFENSE OF A LARVAL TORTOISE BEETLE

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Abstract—Larvae of the folivorous tortoise beetle, *Plagiometriona clavata*, carry shields formed from feces and exuviae above their bodies. We used an ecologically relevant predatory ant, *Formica subsericea*, in a bioassay to determine if shields functioned as simple barriers, as previous studies indicated, or whether they were chemical defenses. Shields were necessary for larval survival; shield removal rendered larvae vulnerable. Shields produced by larvae reared on a substitute diet failed to provide protection. Solvent-leached shields also failed to deter ants, indicating the shield had a host-derived chemical component likely located in the feces, not in the exuviae. *Solanum dulcamara*, the larval host plant, contained free phytol, steroidal glycoalkaloids, and saponins. Shields contained partially deglycosylated metabolites of host steroidal glycoalkaloids and saponins, a suite of fatty acids, and derivatives of phytol, which together formed a deterrent barrier against ant attack. We compared the mobile shield of *P. clavata* to the stationary shield of another *S. dulcamara*-feeding leaf beetle, *Lema trilinea*. Both larval shield defenses were formed from a very similar array of host-derived compounds with deterrent properties. We concluded that convergent patterns of limited chemical transformation and selective incorporation of particular deterrent metabolites in shield defenses of two unrelated taxa represented responses to selection from invertebrate predators.

Key Words—*Plagiometriona clavata*, *Lema trilinea*, *Solanum*, steroidal

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glycoalkaloid, phytol, saponin, fatty acid, fecal shield, larval defense, Chrysomelidae.

INTRODUCTION

Members of the phytophagous leaf beetle family Chrysomelidae exhibit a diverse array of morphological and behavioral attributes for defense against predation. Among these, glandular secretions, maternal care, thanatosis, crypsis, stridulation, and mimicry, to name a few, have been investigated in some detail (Pasteels et al., 1988, and references therein; Schmitt, 1994; Hilker, 1994; Windsor and Choe, 1995; Hespenheide, 1996). However, another putative defensive strategy, larval waste retention, remains poorly understood, although it is a remarkably widespread habit within the Chrysomelidae. For example, nearly 20% of the described chrysomelid species, most notably the members of the Camptosomata, Hispinae, and Criocerinae, have some form of fecal covering or feces-laced structure. Despite their ubiquity, the defensive efficacy of waste-based structures, or fecal shields, as these appurtenances are collectively termed, has yet to be demonstrated in the vast majority of cases (Blum, 1981, 1994; Pasteels et al., 1988; Olmstead, 1994). Available evidence from the few species studied in depth supports the hypothesis that shields, scatoshells, fecal cases, and other waste-based characters function primarily as nonchemical, barrier-type defenses, despite the fact that many species have diets rich in secondary chemicals (Eisner et al., 1967; Erber, 1988; Root and Messina, 1993; Olmstead and Denno, 1993). Fecal-based structures warrant further investigation because they may form more complex defenses than is presently appreciated, perhaps through the incorporation of host-derived or autogenously synthesized compounds.

Within the great variety of fecal attributes displayed by chrysomelid beetles, the shields of larval tortoise beetles, members of the cassidoid Hispinae (sensu Reid, 1995), are perhaps the most intriguing. Attached to a highly movable appendage (furca) emanating from the 8th abdominal segment, and held like a parasol above the larva, the shield is a composite structure made of accumulated feces and of exuviae or molted skins. A remarkable telescoping anus, formed by extrusion of the invaginated 9th and 10th abdominal segments, is capable of precisely depositing fecal material over the shield (Figure 1A). Long suspected to have a defensive function (Riley and Walsh, 1868), the shields of several species examined to date do provide protection by acting as mechanical barriers against some attacking enemies (Eisner et al., 1967; Olmstead and Denno, 1993; Olmstead, 1996).

We report here on the larval shield of the abundant and widespread tortoise beetle, *Plagiometriona clavata* (Fab.), which feeds on woody nightshade, *Solanum dulcamara* L. (Solanaceae), throughout North America. We suspected

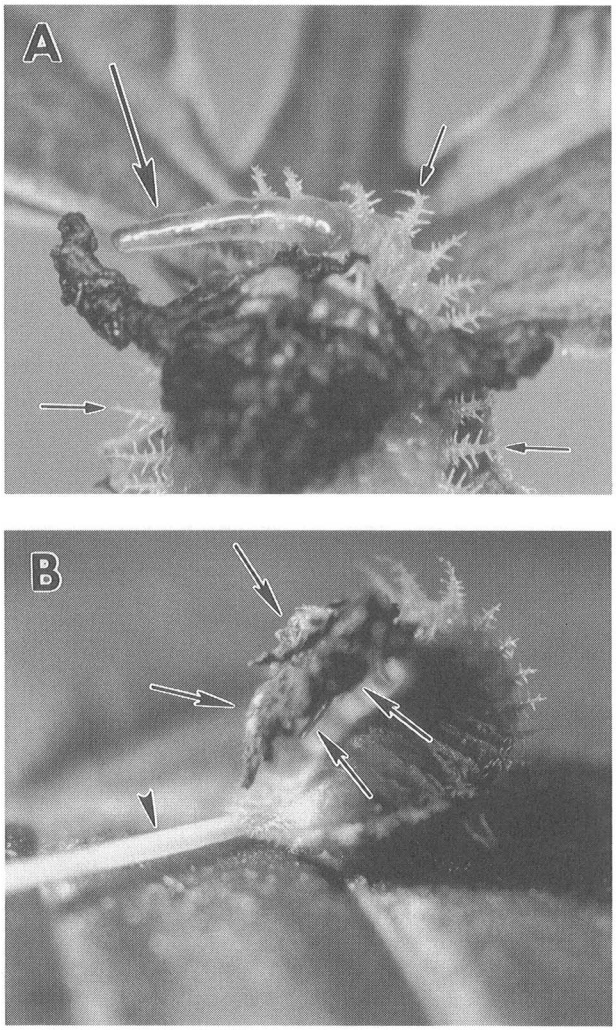


FIG. 1. (A) Larval *P. clavata* (dorsal aspect) with a fecal shield (dark mass) held aloft. Mechanosensitive scoli (small arrows) fringe the larva's flanks. The telescoping anus—the evaginated 9th and 10th abdominal segments—is visible as the translucent tube (arrow), extended to a length of 4.5 mm, and about to apply a bolus of feces to the shield's right projection. (B) Response to perturbation of a *P. clavata* larva with a solvent-leached, experimentally reattached shield. The abdomen is arched over the larva's back, and the chemically modified, reattached shield (bounded by arrows) is aimed forward and is rapidly flagged toward a tactile stimulus, in this instance, by a cat whisker (arrowhead) touching the larva's anterior scoli. This behavior is typical of larvae with unmanipulated shields.

shields might be chemical defenses because *S. dulcamara* is well known to contain many potentially toxic, complex secondary compounds, including steroidal glycoalkaloids (SGAs) and steroidal saponins (Mathé and Mathé, 1979; Tingey, 1984; Roddick, 1986; Hegnauer, 1992). Although a chemical component was suspected in one species (Eisner et al., 1967), the first evidence in support of the idea that shields were chemical defenses appeared only recently (Gómez, 1997; Morton, 1997). The possibility clearly existed that *P. clavata* larvae take advantage of the allelochemicals available in their diet.

Our objectives in this study were fourfold. Firstly, we wanted to know whether *P. clavata* shields provided effective protection from predators, as was indicated by preliminary behavioral observations in the field. Secondly, we wanted to know whether there was a chemical component to what evidently was, according to earlier studies, a simple barrier defense. Thirdly, if shields were found to be chemically deterrent or repellent, we wanted to determine to what extent they were dependent on host-derived compounds. We previously investigated another *Solanum*-feeding leaf beetle, *Lema trilinea* (Criocerinae), and found its shield to be a very effective chemical defense that contained derivatives of the noxious compounds found in the host. Fourthly, we hoped a comparison between *P. clavata*'s mobile, parasollike shield and *L. trilinea*'s static, fecal mound-type shield might reveal convergent metabolic mechanisms for handling inimical host allelochemicals—a comparison of unrelated species, occupying the same trophic niche, afforded us an opportunity to infer whether or not particular chemical or morphological shield features evolved as defensive adaptations or if they were fortuitous by-products of digestion and elimination.

METHODS AND MATERIALS

Collection and Rearing of Insects. Nine adults and a small number of eggs of *P. clavata* were collected from wild populations of *S. dulcamara* in Setauket, New York, on June 10, 1995. They were transported to Penn State University and were reared in cages in a walk-in growth chamber on *S. dulcamara* obtained from root stocks (V&J Seed Farms, Woodstock, IL) and grown in a greenhouse. Shields were collected with fine forceps by slowly teasing the complex of feces and exuviae off the urogomphi of third or fourth instars. This procedure did not harm the larvae (cf. Olmstead and Denno, 1993). Several 2-g samples were collected, immediately weighed, and stored until processed at 4°C in 20 ml of 80% methanol (MeOH). For comparison, we extracted 200 g of the *S. dulcamara* leaves used to feed *P. clavata*.

Bioassays of Larvae by Ant Attacks. Larvae (N = 135), collected as first instars from a host population growing in Crystal Brook Park, Mt. Sinai, New York, during June 1997, were divided into five groups for use in bioassays.

Larvae in all groups were reared under natural light (14L : 10D) and ambient temperature conditions to the third instar (mean length = 4 ± 0.3 mm and with three visible exuviae; N = 20) on 50-cm branches of *S. dulcamara* inside 2-gallon ZipLoc bags. The third instar was selected for assay because it was shown to suffer intermediate levels of predation in two other tortoise beetle species of (cf. Olmstead and Denno, 1993). Twenty-seven larvae had their shields removed with soft forceps 3 hr before being assayed. Shield removal did not harm larvae (Olmstead and Denno, 1993). Shields were removed from 10 additional larvae (not used in assays) and all survived to pupation. A second treatment group (N = 40), reared as above, underwent shield removal. Of these, 24 had their shields soaked in MeOH, while a third group (N = 16) had their shields soaked in water for 24 hr prior to assay. Shields were air-dried for 2 hr on filter paper before being reattached to the urogomphi with white, all-purpose Elmer's glue. Shields retained their wedge shape throughout this procedure. Larvae were checked prior to assay by gentle prodding to guarantee that they could move their shields directionally (Figure 1B). The fourth treatment group (N = 32) was reared on a diet of lettuce until the larvae reached the average size of third instars. Lettuce was previously shown to lack the compounds we found here to be of importance in shields (Morton and Vencl, 1998). Since their developmental rates were slow and some mortality occurred, 16 were assayed as a group when individuals reached the criterion length of ≥4.0 mm. As a control, third instars (N = 26) were assayed with intact, host-derived shields.

An assay trial consisted of the presentation of a single larva, randomized between shielded and treatment groups, in an arena consisting of a sheet of a 3 cm^2 waxed weighing paper pinned to the ground near a foraging trail running near the nest of the sympatric generalist predatory ant *Formica subsericea*. A trial lasted 5 min, or until a test larva was removed from the arena. Time to removal was recorded as was the number of ants contacting a larva. An assay was scored only if a larva was encountered at a rate of ≥2 ants/min.

Extraction and Fractionation. Procedures were the same as presented in Morton and Vencl (1998) and are summarized as follows. An 80% MeOH extract was reduced in volume under vacuum to remove MeOH. The aqueous solution was acidified and extracted with organic solvent (apolar fraction A), then made alkaline and again extracted with organic solvent (alkaline, alkaloidal fraction B). The remaining solution was taken to dryness and the residue was dissolved in methanol (polar fraction C). Fraction A was further purified to remove pigments to the acetonitrile phase by partitioning between hexane and acetonitrile. Fraction B was chromatographed on a silica gel solid phase extraction (SPE) column. The extract was loaded in chloroform ($CHCl_3$) solution, rinsed with ethyl acetate to remove pigments, and the components eluted with propanol. Fraction C was chromatographed on the same SPE silica column after removal of MeOH, loaded in 80 : 20 $CHCl_3$–MeOH and the components eluted with propanol.

Thin-Layer Chromatography (TLC). TLC was used to monitor purity of the B and C fractions through the extraction and fractionation processes. The TLC plates used were plastic-backed silica gel with fluorescent indicator. Alkaloid aglycones were resolved using a modified dichloromethane–acetone (4 : 1) solvent system (Hunter et al., 1976) with two drops of concentrated ammonium hydroxide (NH_4OH) was added to the chamber to improve separation. Alkaloid glycosides were resolved by a $CHCl_3$–EtOH–1% aqueous NH_4OH (2 : 2 : 1 bottom phase) system (Boll, 1962). Alkaloids were detected with Dragendorff's spray reagent (Sigma). Plates were also monitored by long- and short-wave UV.

Gas Chromatography (GC). GC was used to monitor fraction A. An HP 5890 system with a 60-m DB-5 column, FID detector, and Shimadzu CR-4A integrator was used. Run parameters included split injection mode; temperature programmed from 80 to 170°C at 20°C/min from 170 to 225°C at 3°C/min; from 225 to 285°C at 20°C/min with a 10-min final hold; He carrier gas flow rate 2.0 ml/min; injector temperature of 250°C; and a detector temperature of 280°C.

Gas Chromatography–Mass Spectrometry (GC-MS). Fraction A was run on an HP 5890 gas chromatograph equipped with a 30-m DB-5 column, and the column output was applied to an HP 5970 series mass selective detector (GC-MS). The GC operating parameters were as follows: splitless injection mode; temperature programmed from 40 to 250°C at 4°/min with 4-min initial and 20-min final holds; He carrier gas at 1.9 ml/min, with an injector temperature of 250°C. Spectra were statistically compared with the Wiley/NBS Mass Spectral Registry (McLafferty and Stauffer, 1989) by computer. GC-MS results were obtained by the injection of extract from 20 fecal shields.

Fast Atom Bombardment–Mass Spectrometry (FAB-MS). SPE purified fractions B and C were analyzed by FAB-MS. Samples were dissolved in a glycerol matrix and inserted via probe into a Kratos MS 50TC instrument (positive mode, Xenon) and analyzed with the MASSPEC data system.

Data Analysis. Assays of larvae, reported as frequencies (taken as prey or not taken as prey), were analyzed with the Mann-Whitney *U* test (Sokal and Rohlf, 1995).

RESULTS

Shield Bioassays. When *P. clavata* larvae were subjected to attacking ants, the presence of an intact, host-derived shield provided an effective defense against the natural generalist predator we used for an assay (Figure 2; solid bar). If shields were entirely removed, larvae readily fell prey to the ants (Figure 2; hatched bar). To be effective, however, shields had to be produced by larvae reared on a diet of *S. dulcamara*. Shields failed to provide protection if they were derived from a benign substitute diet of lettuce (Figure 2; open bar). Although

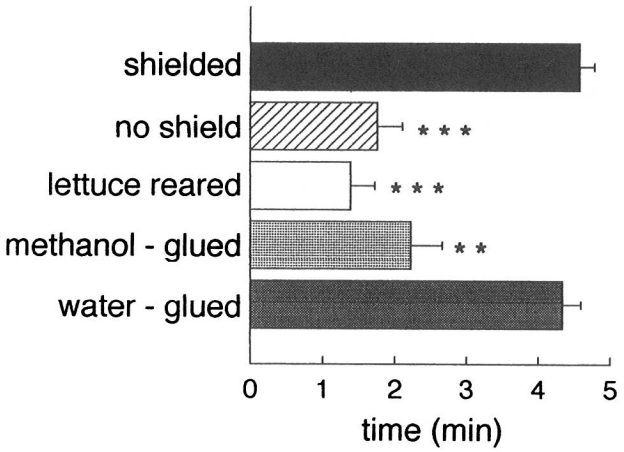


FIG. 2. Effectiveness of intact and modified *P. clavata* shields in a bioassay with the predatory ant, *Formica subsericea*. Solid bar: larvae with host-derived shields (control, $N = 26$); hatched bar: larvae without shields ($N = 27$); open bar: larvae reared on lettuce ($N = 16$); stippled bar: larvae with their shields removed, placed in methanol, and then reattached ($N = 24$); gray bar: larvae with shields removed, soaked in water, and reattached (controls, $N = 16$). Data are given as mean ± SE. Probabilities were calculated with the Mann-Whitney U test; significance levels: $^{**}P < 0.01$; $^{***}P < 0.001$.

able to fend off ants for almost 2 min, larvae with intact, mobile, but lettuce-based fecal shields eventually fell prey to predators. Assay results with micro-modified shields further implicated the importance of a chemical component. When mechanically removed, solvent-leached (MeOH), and reattached to the larval urogomphi, shields lost a significant amount of their effectiveness, despite the fact that micromanipulated, host-reared larvae could directionally maneuver their reattached shields as accurately as the untreated and water-soaked larvae could (Figure 2; stippled bar; Figure 1B). Host-derived shields maintained their protective effectiveness even after experimental removal, soaking in water and reattachment (Figure 2; gray bar). The active chemical component(s) was most likely contained in the methanol-soluble phase of host-derived shields.

Chemical Composition of Fraction A. Analysis by GC-MS of nonpolar fraction A resulted in 31 peaks with sufficient information for their partial or full characterization (Figure 3; Table 1). There were significant amounts of 17 fatty acids (FAs), ranging in length from 14 to 18 carbons. Although many were detected as methyl esters, the largest FA components, hexadecanoic (palmitic) acid (peak K) and 9-octadecenoic (oleic) acid (peak T), were present as free acids, which in combination, accounted for 31.6% of the total A fraction profile. FAs and their methyl esters comprised 48.8% of fraction A.

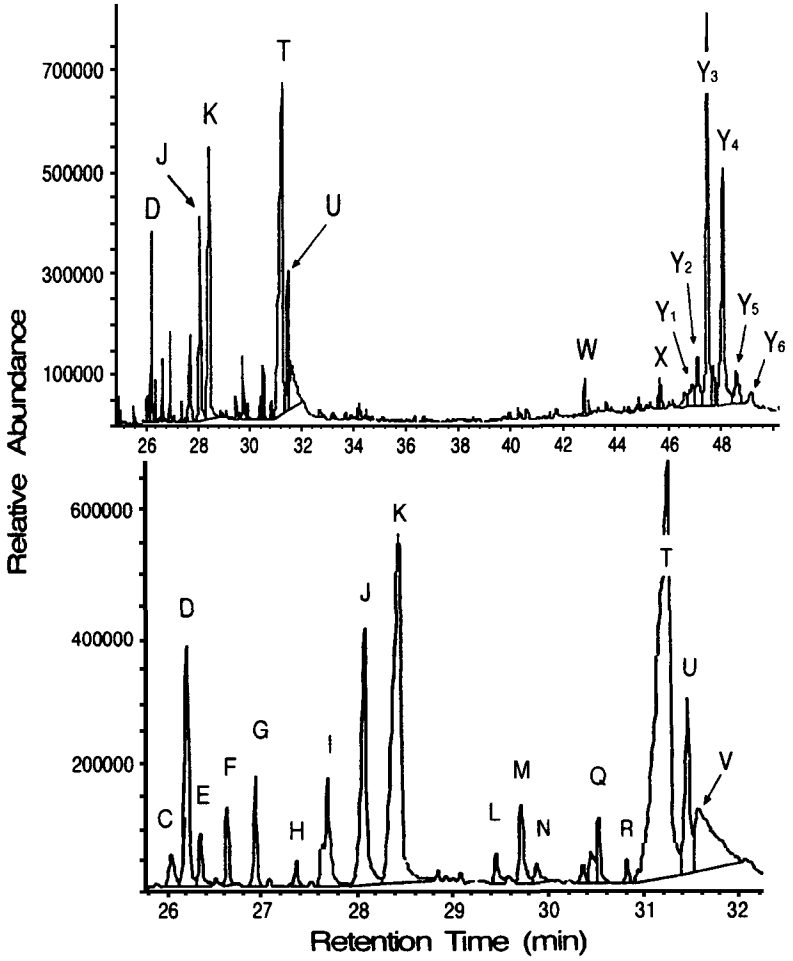


FIG. 3. Separation of compounds by GC-MS in the apolar fraction A of *P. clavata* shields. Top: entire total ion count spectrum. Bottom: expanded region of the congested area between 26 and 32 min from the upper trace. Lettered peaks are identified in Table 1.

Fraction A also included three uncharacterized phytol derivatives (Figure 3; peaks F, G, V). They likely represent degradation products of free phytol found in the plant and total about 11% of the A profile. The sapogenin, diosgenin (peak Y_1), and five related derivatives (peaks Y_2 to Y_6; Figure 3; Table 1) are metabolites of the host-plant steroidal saponin, dioscin (dioscin-chacotrios), that together accounted for 38% of fecal fraction A. The different retention times

TABLE 1. IDENTIFICATION OF GC-MS SPECTRUM FROM *P. clavata* SHIELD FRACTION A[a]

Peak	Retention time (min)	Compound	Mol. wt.	*Plagiometriona clavata*
A	24.96	tetradecanoic acid	228	0.7 (97)
B	25.53	tetradecanoic acid ethyl ester	256	0.3 (99)
C	26.05	pentadecanoic acid	242	0.6 (97)
D	26.22	phytol derivative	278	3.5
E	26.36	6,10,14-trimethylpentadecan-2-one	268	0.7 (91)
F	26.64	phytol derivative	278	0.9
G	26.94	phytol derivative	278	1.4
H	27.37	9-hexadecenoic acid methyl ester	268	0.4 (99)
I	27.70	hexadecanoic acid methyl ester	270	2.5 (98)
J	28.10	9-hexadecenoic acid	254	5.5 (94)
K	28.46	hexadecanoic acid	256	11.6 (99)
L	29.46	heptadecanoic acid methyl ester	284	0.4 (78)
M	29.73	heptadecanoic acid	270	1.3 (91)
N	30.37	9,12-octadecadienoic acid methyl ester	294	0.3 (99)
O	30.46	9-octadecenoic acid methyl ester	296	trace (91)
P	30.48	9,12,15-octadecatrienoic acid methyl ester	292	0.6 (76)
Q	30.54	11-octadecenoic acid methyl ester	296	0.9 (99)
R	30.83	octadecanoic acid methyl ester	298	0.3 (98)
S	31.04	9,12-octadecadienoic acid	280	trace (96)
T	31.27	9-octadecenoic acid	282	20.0 (99)
U	31.47	octadecanoic acid	284	3.4 (99)
V	31.58	phytol derivative	284	4.6
W	42.81	hexatriacontane	507	0.8 (94)
X	45.68	tigogenone	414	0.8 (94)
Y_1	46.65	diosgenin	414	0.9 (94)
Y_2	47.15	diosgenone	412	2.7
Y_3	47.59	diosgenone metabolite	412	18.0 (97)
Y_4	47.73	diosgenone minus 2-H	410	1.3
Y_5	48.16	diosgenone minus 2-H	410	12.7
Y_6	48.62	diosgenone minus 2-H	410	2.3

[a]Peak labels are those designated in Figure 3. Data for each peak include: retention time, identity, molecular weight, percentage of total integrated peak area, and confidence in the match with library standards (%).

of the diosgenin derivatives, with m/z 410, are possibly due to the addition of double bonds at various locations.

Chemical Composition of Fraction B. After partitioning of apolar compounds into fraction A, the fractionation scheme was designed to separate alkaloids into fraction B, while leaving polar substances in fraction C. However, due to their high polarity, the steroidal glycoalkaloids (SGAs) of *S. dulcamara* parti-

tioned between both fractions B and C. FAB-MS of the *P. clavata* fecal fraction B contained hydroxylated aglycones with differing numbers of double bonds (m/z 426, 428, and 430; Figure 4). By isolating and weighing the compounds from 2.0-g aliquots ($N = 2$) of feces, the concentration of aglycone metabolites in *P. clavata* feces was estimated to be approximately 0.65 mg/g fresh wt.

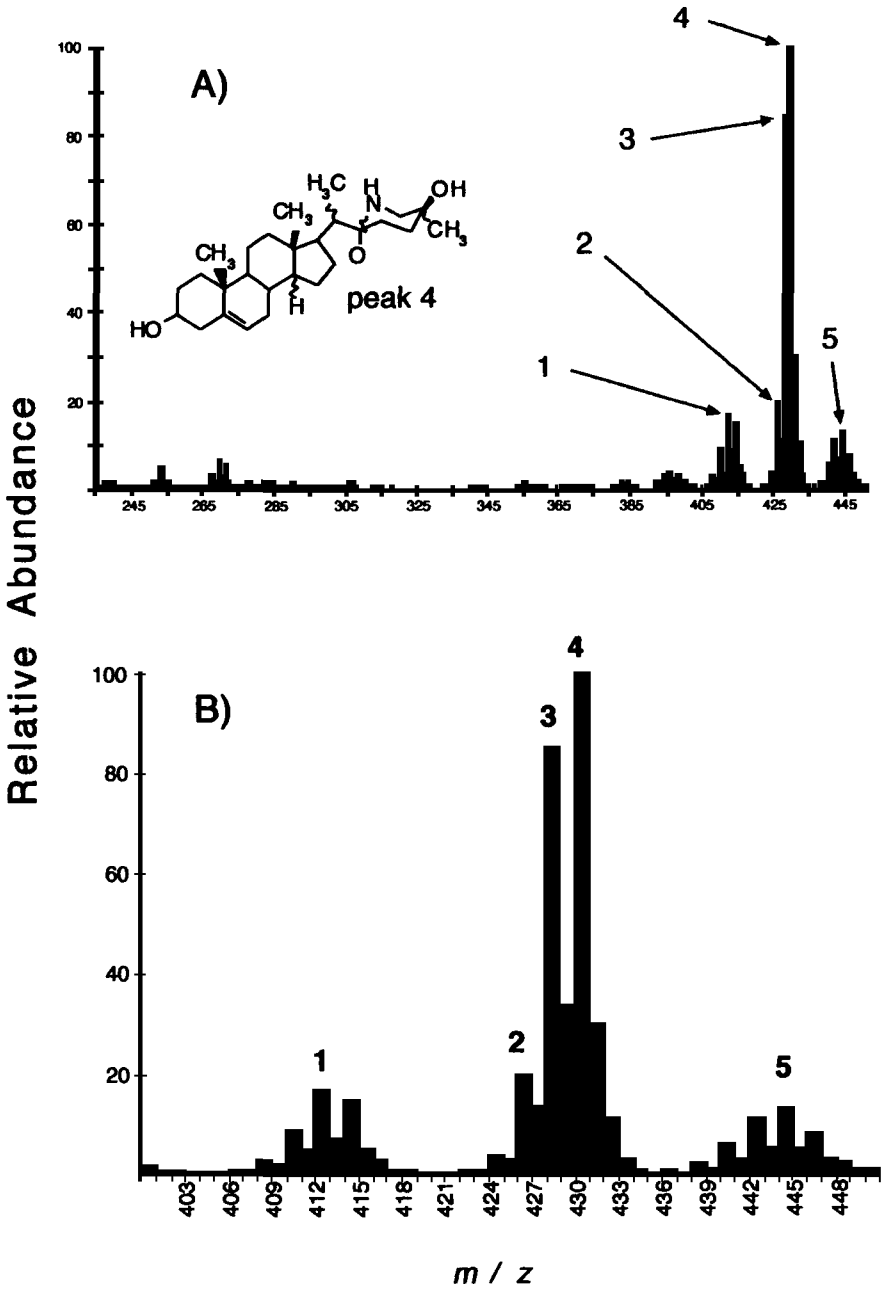
Comparison of Host Plant and Shield C Fractions. Figure 5 shows the FAB-MS fragmentation spectrum of SGA metabolites in the C fractions of host plant and of fecal shields. Structures were identified by comparison with fragmentation patterns of published spectra for *Solanum* SGAs (Price et al., 1985); each predicted fragment ion of the solasodine glycosides, α-solasonine and α-solamargine, were observed in the spectra from *S. dulcamara* (Figure 5A).

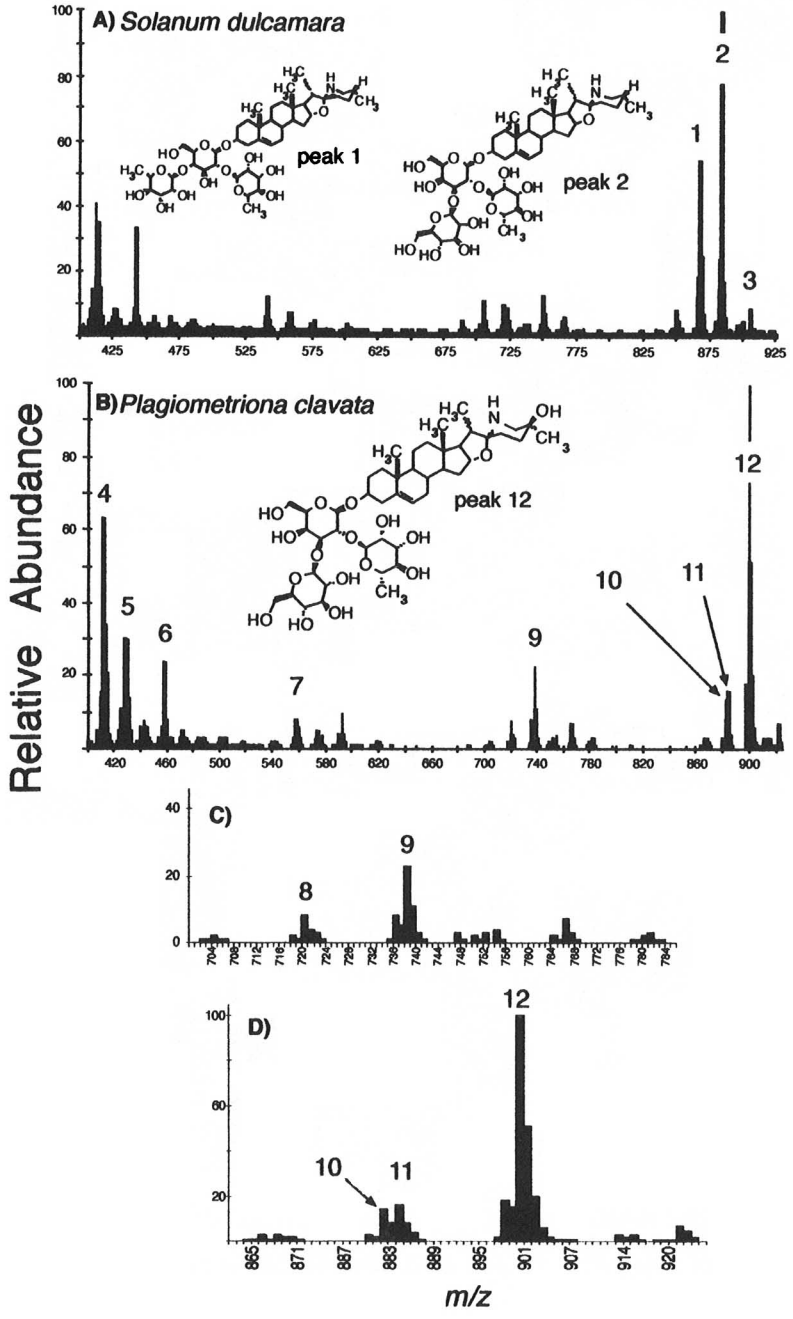
Plagiometriona clavata did not remove all the sugars from all the host plant's SGAs during transit through its gut. The fecal fraction C contained SGA metabolites with molecular ions and fragments 16 mass units higher than those for the host's SGAs, indicating that the insect can efficiently hydroxylate dietary SGAs but is evidently less efficient at deglycosylating them. We assume that this hydroxyl function is the same one observed on the aglycones at carbon 26 and that hydroxylation of the F ring represents the first step in SGA metabolism, which may prevent SGA membrane insertion, thereby preventing their toxic effects. The concentration of hydroxylated SGAs in *P. clavata* shields was estimated to be approximately 0.1 mg/g fresh wt.

DISCUSSION

Shield Bioassays. The bioassay clearly established that larval shields provided an effective defense against a natural predator of *Plagiometriona clavata*. Because of their parasol-like location above the larvae, *P. clavata*'s shields afforded a unique opportunity to analyze experimentally how they functioned as defenses without harming the larvae. Shields were necessary for larval survival, because without them, larvae fell prey to the ubiquitous generalist predatory ant that we used in the bioassay and which is found in *P. clavata*'s habi-

FIG. 4. (A) FAB-MS fragmentation spectrum of fraction B from *P. clavata*'s shield and the proposed structure of the most abundant metabolite. The main SGA metabolite, corresponding to peak 4, is an hydroxylated aglycone of the host-plant's SGA. (B) Expanded region, from m/z 400 to m/z 450, of fraction B from *P. clavata*'s shield. The cluster of molecular ions with $m/z+16$ mass units may represent hydroxylated forms of the main SGA metabolites surrounding peak 4. The cluster of ions with $m/z - 18$ mass units are fragments (minus water) of each of the ions within the main SGA cluster. The aglycone, solasodine (mol. wt. 413) is present as the small peak with m/z 414.


A)
B)
peak 4
Relative Abundance
m / z
1
2
3
4
5


A) Solanum dulcamara
peak 1
peak 2
1
2
3
B) Plagiometriona clavata
peak 12
4
5
6
7
9
10
11
12
Relative Abundance
C)
8
9
D)
10
11
12
m/z

tat. To determine whether shields function primarily as physical barriers or as chemical defenses, we modified shield chemical content, either by altering the larval diet or by treating them with solvents. In the diet-modification experiment, larvae without access to host compounds were defenseless. Not only did their shields lack the dietary compounds ordinarily obtained from the natural host, larvae reared on the substitute diet were unable to synthesize any defensive compounds of their own. The micromanipulation experiment, consisting of shield removal, solvent-soaking, and reattachment, demonstrated that the chemical component resided in a methanol-soluble phase. This implied that the mere presence of a shield, if devoid of host-derived compounds, was insufficient to protect its bearer. These observations lent additional support to the hypothesis that *P. clavata*'s shield is a chemical defense, rather than a physical barrier.

Larval chemical defenses have been hypothesized to contain volatile repellents that thwart invertebrate predators before they can contact and damage the soft larval cuticle (Eisner and Meinwald, 1966; Pasteels et al., 1983, 1984). By providing an effective defense against predation, the shields of *Eurypedus* and *Coptocycla* species, tropical tortoise beetles feeding on *Cordia* (Boraginaceae), were essential for larval survival (Gómez, 1997). The effectiveness of *E. nigrosignata*'s shield rests on volatile repellents (mono- and diterpene essential oils) acquired from the host plant and discharged unchanged in feces (Gómez, 1997). In contrast, our bioassay indicated that *P. clavata*'s shield owed its effectiveness to deterrents, rather than repellents; ants attacked larvae, but if they contacted shields, they retreated and groomed vigorously. This observation allowed us to focus our search on the nonvolatile constituents in *P. clavata*'s shield.

Origin, Identity, and Deterrence of Shield Compounds. Solanum species are well known to contain many complex and toxic secondary compounds, including steroidal glycoalkaloids and steroidal saponins (Roddick, 1986; Hegnauer, 1992). The leaves of *P. clavata*'s host, *S. dulcamara*, are also a rich source of

FIG. 5. Comparison of FAB-MS spectra from fractions C from *S. dulcamara* and from *P. clavata*'s shield. (A) Host plant. Structure at left, corresponding to peak 1, is α-solamargine (solasodine-chacotriose). Structure at right, corresponding to peak 2, is α-solasodine (solasodine-solatriose). Peak 3 is the sodium adduct (MNa^+) of peak 2 with m/z 906. (B) Fecal shield. The proposed structure of the SGA metabolite, corresponding to peak 12, is hydroxylated at carbon 26 of the F ring (peak 12, m/z 900). (C) The expanded region of *P. clavata*'s fraction C from m/z 700 to m/z 786. (D) The expanded region of *P. clavata*'s fraction C from m/z 801 to m/z 928. There is no evidence for the addition of double bonds prior to the removal of sugars, as there was for fraction B, which indicates that the first steps in SGA metabolism are hydroxylation followed by deglycosylation. The remaining ions are SGA fragments; each insect metabolite is 16 mass units greater than the equivalent SGA fragment.

free phytol, in addition to steroidal saponin, dioscin, and two glycosides of the SGA solasodine (Morton and Vencl, 1998). Data from host fraction C further confirmed the SGA component to be a mixture of two glycosides of solasodine that differed only in the configuration of their trisaccharide moieties.

Plagiometriona clavata's shield contained four major classes of compounds: (1) SGA derivatives, (2) a suite of dioscin metabolites, (3) FAa, and (4) phytol derivatives. With the possible exception of the FAs, which may have been liberated from plant triglycerides, all shield metabolites had host plant precursors. Representatives of the four compound classes, or their closest commercially available analogs, were previously demonstrated to be deterrent to ants (Morton and Vencl, 1998; Vencl and Morton, 1998), and we cite additional information justifying their functional designation as deterrent compounds.

The steroidal components of *P. clavata*'s shield were derived from the host's α-solasonine (solasonine-solatriose) and α-solamargine (solasodine-chacotriose), while the diosgenin derivatives were metabolites of the plant's saponin, dioscin (dioscin-chacotriose). The specifics of steroid metabolism will be discussed below. Steroidal alkaloids and saponins are well known to have significant toxic effects (Appelbaum and Birk, 1979; Harborne, 1986; Keukens et al., 1996; Mitchell and Harrison, 1985; Roddick, 1986; Roddick et al., 1990). However, with the exception of the leaf beetle shields discussed below, there are no other reports of insects either metabolizing or of using these steroids for defense (Brown, 1987); their presence in *P. clavata*'s shield is exceptional.

The third shield component, the FAs, accounted for nearly half (49%) of the total fraction A chemical profile. In contrast to the other shield components, there was a discrepancy between the ingested host FAs and the shield FA content. Neither the main shield FAs, octadecenoic (oleic) acid and hexadecenoic (palmitoleic) acid, nor a variety of minor FA methyl esters were detected in the host plant. Since the FA compositions of tortoise beetle shields and of leaf beetle body extracts are similar (Thompson, 1973), some shield FAs may have come from the larvae themselves. However, it is more likely that larvae liberated FAs from phospholipids and triglycerides of the plant. Since bound FAs do not extract into the 80% methanol solution, unbound FAs would not be detectable in the plant with the extraction and fractionation protocol employed here.

In contrast to SGAs and saponins, FAs are found in other invertebrate defenses. For example, unsaturated FAs, such as oleic and methyl palmitate, are also present in chrysomelid eggs and in the defensive secretions of wasps, where they act to deter ants (Howard and Tschinkel, 1975; Pasteels et al., 1986; Eisner et al., 1996; Dani et al., 1996). Several other FAs, including the ones identified in shields, evoke necrophoresis (undertaker) behavior (Blum, 1970; Haskins, 1970; Howard and Tschinkel, 1975; Hölldobler and Wilson, 1990) in members of the Formicidae.

Lastly, the defensive potential of unbound phytol and its derivatives has

been discussed elsewhere (Morton and Vencl, 1998). For example, 6,10,14-trimethylpentadecan-2-one, one of the phytol derivatives identified here, was found to be part of the defensive secretion in at least one species of formicine ant (Regnier and Wilson, 1968). Thus, the presence in shield defenses of compounds demonstrably capable of altering ant behavior, such as SGAs, diosgenin, FAs, and phytol derivatives, clearly seems of functional benefit to tortoise beetle larvae.

Adaptive Convergence of Solanum*-Derived Fecal Shields.* Whenever similar character states evolve independently in similar environments, one can ask if they were adaptations that benefit their bearers. Comparisons of convergent characters, especially if they are complex, can be useful for the identification of particular agents of contemporary selection that maintain them as functionally beneficial adaptations. We compared two unrelated fecal shield-bearing taxa occupying the same trophic niche.

The fecal shield of the shining leaf beetle, *Lema trilinea*, which also feeds on *S. dulcamara*, is an effective defense necessary for larval survival that is based on host-derived chemicals (Morton and Vencl, 1998). Besides the elaborate anatomical modifications for fecal deposition above the larvae, there were several striking chemical parallels between the shield systems of both species. For example, the profile of FAs, especially the large amount hexadecanoic acid, was similar. Although there is more oleic acid in the tortoise beetle's feces than occurred in *L. trilinea*'s, the suite of diosgenin derivatives is virtually identical for both species in both their amounts and their MS fragmentation patterns.

The most interesting similarity between shield systems is how the two beetle species transform and used the SGAs and saponins, potentially the most inimical allelochemicals they encounter. To compartmentalize and eliminate allelochemicals from the organism, metabolic detoxification systems typically increase allelochemical polarity (i.e., aqueous solubility) by using some combination of hydrolysis, oxidation, conjugation with a polar moiety, or reduction. (Dauterman and Hodgson, 1978; Duffey, 1980). As noted, SGAs and saponins are considered extremely toxic; they can bind with membrane-bound sterols, thereby altering membrane permeability or causing loss of cell integrity (Keukens et al., 1996; Mitchell and Harrison, 1985; Roddick et al., 1990). SGAs and saponins differ only by the substitution of nitrogen with oxygen in their respective F rings, and they produce their toxic effects by insertion of their apolar F-ring portions into the target membrane. Both beetles evidently use a three-step hydrolysis–reduction strategy to detoxify and then "sequester" SGAs in their shield defenses. They first hydroxylate, then deglycosylate, and finally add extra double bonds and additional hydroxyls to both SGA and diosgenin residues. Hydroxylation of the apolar portions of saponins and SGAs might well reduce their membrane binding tendency at the same time it increases their polarity, making them more readily confinable within the gut and facilitating their elimi-

nation in feces. SGA-laced feces, not accidentally we think, become strategically positioned on or above larvae, to act as deterrents in shield defenses.

CONCLUSIONS

Instead of being metabolized, erstwhile nutrients and some toxic secondary compounds formed part of the larval shield. The particular metabolites we identified were likely not incidental digestive by-products, but probably represented adaptations for defense. The presence of particular compounds in shields was most plausibly explained by their deterrence, which evidently outweighed their nutritional value. Before the sufficiency of this explanation was assumed, however, three sources of evidence provided by this study supported the idea that shields, and the specifics of their chemical makeup, are adaptations maintained as selective responses to predation: (1) shields are necessary for larval survival; (2) individual compounds have beneficial effects, i.e., they act as deterrents, and (3) shields convergently function as chemical defenses in species with the same ecology. In the limit, the inclusion of host-derived allelochemicals in shields may be due to the apparent inability of larvae to synthesize their own defensive compounds. Nonetheless, defense is perhaps the best explanation for the occurrence of particular compounds in shields.

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VOLATILES OF BRACKET FUNGI *Fomitopsis pinicola* AND *Fomes fomentarius* AND THEIR FUNCTIONS AS INSECT ATTRACTANTS

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Abstract—Volatiles released from fruiting bodies of the polypores *Fomitopsis pinicola* and *Fomes fomentarius* (Polyporaceae) were collected by entrainment and SPME techniques and analyzed by GC-MS. The most significant difference between the two species was found in the terpene fraction. *F. fomentarius* emitted a more complex blend, with more than 10 sesquiterpenes. During the sporulating phase, the release of (*R*)- and (*S*)-oct-1-en-3-ol, octan-3-one, and some sesquiterpene hydrocarbons (mainly β-barbatene) increased in *F. pinicola*, whereas in *F. fomentarius* the release of octan-3-one, *cis*-furanoid linalool oxide, β-phellandrene, β-myrcene, and several sesquiterpene hydrocarbons increased. β-Barbatene was identified for the first time in fungi. Chopping of the fruiting bodies altered the odor composition more in *F. fomentarius* than in *F. pinicola*. Five volatiles giving a typical fungal odor (*rac*-oct-1-en-3-ol, nonan-1-ol, *rac*-octan-3-ol, octan-1-ol, and octan-3-one) were tested for insect attraction in the field. Females of the three wood-living generalist beetles *Malthodes fuscus*, *Anaspis marginicollis*, and *A. rufilabris* and both sexes of the moth *Epinotia tedella* were attracted to *rac*-oct-1-en-3-ol. The generalist predator on fungus-insects *Lordithon lunulatus* was attracted to *rac*-oct-1-en-3-ol and octan-3-one in combination. Previous results regarding the host-specific attraction of beetles to the odors emanating from chopped fruiting bodies of *F. pinicola* and *F. fomentarius* are discussed in the light of this investigation.

Key Words—*Fomitopsis pinicola*, *Fomes fomentarius*, *Epinotia tedella*,

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fungal odor, sporulation, insect attraction, oct-1-en-3-ol, octan-3-one, β-barbatene, sesquiterpene hydrocarbons.

INTRODUCTION

The fruiting bodies of fungi are patchily distributed and constitute a rather unpredictable resource to insects using them as breeding or feeding substrates. As in the case of many herbivores (Bernays and Chapman, 1994), orientation towards host odors can be assumed to be of importance to fungivorous insects colonizing new patches. Fungal odors have been shown to attract insects living on fungi (Pacioni et al., 1991; Bengtsson et al., 1991; Jonsell and Nordlander, 1995) or on substrates decayed by fungi (Honda et al., 1988; Phelan and Lin, 1991; Pierce et al., 1991; Pfeil and Mumma, 1993).

Insects breeding in bracket fungi (Polyporaceae) are generally more host specific than other fungivores (Hanski, 1989). Cisid beetles (Cisidae) monophagous on *Fomitopsis pinicola* Fr. (Karst.) discriminate in flight between the true host odor and the odor of the closely related *Fomes fomentarius* (L. ex Fr.) Kickx. (Jonsell and Nordlander, 1995). This discrimination could be due to single host-specific compounds or to characteristic blends of substances. In this paper we analyzed and characterized the odor composition of these two polypores and suggest how they may be recognized by insects. Both fungi are common in Sweden and many insect species are associated with them (Jonsell and Nordlander, 1995).

In contrast to cisid beetles associated with *F. pinicola*, no attraction to host odor has been detected within the guild of cisid beetles breeding in *F. fomentarius* (Jonsell and Nordlander, 1995). Chopped fruiting bodies were used as an odor source in this study and might explain this difference in attraction. The chopping might have caused different changes in the two species. Furthermore, in order to eliminate individual variations, many chopped pieces were mixed prior to use as baits. If only a few of the fruiting bodies were attractive initially, this may have resulted in the observed lack of attraction.

In other fungi (*Penicillium* and *Aspergillus* species) the production of odoriferous metabolites increases during sporulation (Börjesson et al., 1993). Such changes might be important cues for insects breeding on spores (Hågvar and Økland, 1997). In this study we have analyzed the changes in odor composition over time in individual living fruiting bodies of the two species studied by Jonsell and Nordlander (1995) and also analyzed the changes appearing after chopping, in order to estimate the possible influence of the above-mentioned factors (time and chopping) on the results of the previous attraction study (Jonsell and Nordlander, 1995).

Many beetles living in decaying wood or in various types of fungi are

attracted to the odor of chopped polypore fruiting bodies (Jonsell and Nordlander, 1995). For this reason we tested the attractivity to insects in field experiments of five substances identified in the *F. pinicola* bouquet. These substances occur in many other fungi and can be regarded as common fungal volatiles (Abraham and Berger, 1994; Gallois et al., 1990; Hanssen et al., 1986; Pyysalo, 1976). Thus, they can be expected primarily to attract generalist fungivores that do not require components typical of particular fungal hosts.

METHODS AND MATERIALS

Volatiles from Living Fruiting Bodies In Situ. Odor samples from the bracket fungi *F. pinicola* and *F. fomentarius* were collected from fruiting bodies in situ on tree trunks. The trees were situated in seminatural parts of coniferous and mixed forests, 35–70 km north–northeast of Stockholm, Sweden. The *F. pinicola* materials consisted of four fruiting bodies (100–200 mm diam., three odor samples per fruiting body) growing on the trunks of four dead spruces [*Picea abies* (L.) Karst.] and two large fruiting bodies (180 and 230 mm diam., four odor samples per fruiting body) growing on the same spruce stump. The *F. fomentarius* materials consisted of six fruiting bodies (70–300 mm diam.) on different trunks of dead birch, *Betula* spp.

Seasonal Variation. Seasonal variation in the release of volatiles was studied by means of odor samples taken from the same fruiting bodies on three occasions. Four of the *F. pinicola* fruiting bodies were sampled in June, July, and August, and two in May, June (the 6th and 28th), and October. At each visit we noted whether the fruiting body was sporulating or not.

Odor differences between sporulating and nonsporulating phases also were measured on fruiting bodies growing on eight trunks that had been brought indoors. They were transferred to room temperature (18°C) in January (outdoor temp. −1°C) and kept singly standing in buckets with a small quantity of water. Four of them were spruce trunks (*Picea abies*, 0.4–0.7 m high, 0.2–0.3 m diam.) with one to three fruiting bodies of *F. pinicola*, and four were birch trunks (*Betula* spp., 1.0–1.2 m high, 0.15–0.20 m diam.) with one to three fruiting bodies of *F. fomentarius* (100–150 mm diam.). Headspace air samples were taken before and during the sporulation, by entrainment and the SPME techniques (both methods are described in the section "Sampling of Volatiles"). Between the collection periods the entire trunks were covered in polyethene bags to maintain high humidity.

Chopped Fruiting Bodies. The composition of the volatiles released from odor baits of the kind used by Jonsell and Nordlander (1995) was studied on fruiting bodies of *F. pinicola* and *F. fomentarius* chopped into ca. 8 cm^3 pieces. Batches of 0.5 liters of this material were put into metal tubes (75 × 230 mm,

volume 1.2 liters) with a fine metal wire netting at both ends. The tubes were sealed with plastic bags at both ends before and between the occasions of sampling by the entrainment technique. After chopping, volatile samples were collected for 8 hr on days 1, 7, and 14. The tubes with chopped fungi were kept outdoors between sampling days.

Sampling of Volatiles. The entrainment technique described by Borg-Karlson (1990) was used for sampling the fungal volatiles. The fruiting body was enclosed in a plastic bag (polyethene terephthalate, Look, Meny), through which air was drawn by suction (Ametek 863 suction pump, Aircheck Sampler (SKC) 230 or Aircheck Sampler (SKC) 60, flow rate 100 ml/min). Porapak Q was used as the adsorbent (ca. 100 mg, 60–80 mesh), fixed with glass-wool (Pyrex brand wool) in a glass tube (60 × 5 mm). The Porapak Q was extracted with pentane (pro analysi, Merck) and the first 10 drops (0.5 ml) of extract were collected for the analyses without further concentration. The sorption material in the tubes was cleaned by rinsing with pentane and dichloromethane before and after each entrainment.

As a complement to the entrainment technique, the SPME technique (solid-phase microextraction) (Zhang and Pawliszyn, 1993; Borg-Karlson and Mozuraitis, 1996) was used with two coatings: polydimethylsiloxane (PDMS) 100 μm and Carbowax/divinylbenzene 65 μm (Supelco). Each fruiting body was enclosed in a plastic bag (as described above for the entrainment technique), and the syringe was mounted close to the fungus, to work during an adsorption time of 1 hr with the fruiting bodies brought indoors and 2 hr with the *F. fomentarius* in situ. Before collections, the fiber coating was cleaned by thermal desorption in a GC split/splitless injector (225°C) for 10 min and the result checked by one GC injection.

Analyses of Samples. All samples were analyzed by using a Finnigan SSQ 7000 mass spectrometer connected to a Varian 3400 GC, with electronic ionization (EI). A DB-5 MS fused silica column (J&W Scientific, 30 m, 0.25 mm ID, 0.25-μm coating layer) and a DB-WAX column (J&W Scientific, 30 m, 0.25 mm ID, 0.25-μm coating layer) were used. The temperature program was 40°C (4 min), followed by an increase of 4°C/min to 200°C, remaining at 200°C for 30 min. The injector was of a split/splitless type, closed for 0.5 min at isothermal 225°C. The constituents were identified by comparison of their mass spectra and GC retention values with those of reference compounds and those given in the Finnigan NS library.

A two-dimensional Varian 3400 GC system was used to determine the enantiomeric compositions of oct-1-en-3-ol, *cis*-furanoid linalool oxide, β-phellandrene, and limonene in samples containing sufficient amounts of the compounds. Other chiral constituents were not analyzed because their concentrations were low or samples of one or both of the enantiomers were unavailable. The GC-system was equipped with a split/splitless injector (200°C) and a flame ioniza-

tion detector (225°C) and consisted of two GCs, which were connected with a heated interphase (70°C). The system is described in Borg-Karlson et al. (1993). A DB-WAX fused silica column (J&W Scientific, 30 m, 0.25 mm ID, 0.25-μm film thickness) was used for the nonchiral separations in the first GC. The temperature program was 40°C (4 min), followed by an increase of 4°C/min to 200°C, remaining at 200°C for 30 min. In the second GC, separation of the enantiomers was performed by using two types of columns. The enantiomers of oct-1-en-3-ol were separated on the chiral column Lipodex E (dipentylbutyryl-γ-cyclodextrin, Macherey-Nagel; 25 m, 0.25 mm ID) with the temperature program of 47°C (40 min) followed by 1°C/min to 120°C. *cis*-Furanoid linalool oxide (*cis*-5-ethenyltetrahydro-α,α,5-trimethyl-furan-2-methanol), β-phellandrene, and limonene were separated on a Cyclodex-B (permethyl-β-cyclodextrin/DB-1701, J&W Scientific, 25 m, 0.25 mm ID) column with the following temperature programs: (1) 100°C (40 min) (*cis*-furanoid linalool oxide) and (2) 55°C (11 min), followed by an increase to 75°C with 1°C/min (the monoterpenes). A reference sample of (*R*)-oct-1-en-3-ol was obtained in an ee of 99.9% via an enzymatic resolution (Fäldt et al., unpublished data). (+)-β-Phellandrene was synthesized according to Valterová et al. (1992) and so were the enantiomers of the *cis*-furanoid linalool oxide, which were synthesized recently (Borg-Karlson et al., 1996). Pure enantiomers of limonene were obtained from Aldrich.

Isolation and Structural Determination of β-Barbatene. Chopped *F. pinicola* fruiting bodies (600 g) were extracted in 1.3 liters of hexane at room temperature for 15 min. The extract was dried with $MgSO_4$, the solvent was evaporated, and the remaining crude oil (4.6 g) was subjected to medium pressure liquid chromatography (MPLC). A column (50 cm × 25 mm ID) was loaded with silica gel (12 g, Merck, pore diameter 60 Å, particle size 35–60 μm). The sample was preadsorbed on the same type of gel (8 g) and put on top of the column. For gradient elution, hexane–ethylacetate mixtures (100 ml/portion) were used with increasing percentage of ethylacetate (percentages of ethylacetate per portion: 0; 0.6; 1.25; 2.5; 5; 7.5; 10; 15; 20; 40; 60; 80; 100). The fractions were collected in 25-ml vials (23 ml eluted in each vial).

The main constituent in the first two fractions was a sesquiterpene with GC purity of 75%. The compound was analyzed (15.1 mg in $CDCl_3$) by means of a Bruker AM 400 and a Varian Unity 500 NMR spectrometer and found to be β-barbatene (Andersen et al., 1973; Matsuo et al., 1985; König et al., 1996). The methods used were DQCOSY, NOESY, Heteronuclear single quantum coherence (HSQC) (Bodenhausen and Ruben, 1980; Braun et al., 1996) and Heteronuclear shift correlations via multiple bond connections (HMBC) (Bax and Summers, 1986; Braun et al., 1996). The 1,1-ADEQUATE method (Reif et al., 1996) was used for determining the positions of the quaternary carbon atoms.

Multivariate Data Analysis. GC data given by 31 significant constituents of the volatiles collected from the two fungus species, either by the entrainment

technique in situ or from chopped fruiting bodies, were selected for statistical analysis. The data were evaluated by principal component analysis (PCA) and projections to latent structures–discriminant analysis (PLS-DA), with the program CODEX version 2.6 (Chemometric Optimization and Design for EXperimenters, which was installed as an add-in module to Microsoft Excel). The data were normalized to the total peak area and logarithmic transformations were made. For more details, see Persson et al. (1996). Each variable was scaled to unit variance (autoscaling). The numbers of significant components were determined by cross-validation (CSV/SD) (Wold et al., 1985). A component was judged to be significant, when CSV/SD was ≤ 0.95. In PLS-DA, dummy Y values of 1 and -1, respectively, were used for the two classes of samples (in situ and chopped) (Wold et al., 1985).

Insect Attraction. The attraction of insects to the fungal volatiles was tested in field experiments with baited window traps. The experiments were carried out at a spruce-dominated site in the Lunsen forest, a seminatural coniferous forest, located 70 km north of Stockholm. On that site there was an abundance of dead trees and logs with *F. pinicola* brackets and large populations of insect species associated with wood-decaying fungi (Jonsell and Nordlander, 1995).

The window traps were arranged in blocks. Each block included one replica of each bait to be tested as well as an empty control trap. For the first experiment, four compounds had been selected according to preliminary determinations of volatiles in *F. pinicola*. These four compounds were presented together, and one of them was also presented alone. Twelve blocks were included, and each consisted of three differently baited traps: Mix—a mix of octan-1-ol, *rac*-octan-3-ol, nonan-1-ol, and *rac*-oct-1-en-3-ol (equal amounts in separate vials, all Aldrich 98%); A (alcohol) consisting of *rac*-oct-1-en-3-ol; and C (control) was empty. The trapping period lasted from June 17 to July 12, 1994.

Subsequent chemical analyses revealed that octan-3-one was also an important odor component. Therefore, this compound was included in a second experiment, which was carried out between June 10 and July 29, 1997. Ten blocks were used, each with four differently baited traps: A—*rac*-oct-1-en-3-ol; K (ketone)—octan-3-one; A + K—*rac*-oct-1-en-3-ol and octan-3-one; and C—empty.

The three or four traps of each block were placed in the corners of a triangle or a square with 5- to 10-m sides, and the blocks were placed at least 20 m apart. No dead logs with a diameter larger than 10 cm were allowed closer than 10 m from any trap. The upper part of the trap consisted of a transparent vertical PVC window (0.35 m wide, 0.5 m high), nailed to two wooden poles, with the top of the window 1.5 m above ground. Below the window was a plastic jar with an 18- $\times$ 18-cm opening, where the insects were caught in water with some detergent added. The odor baits were attached in a small opening at the bottom of the window and consisted of polyethylene vials containing 0.5 ml of the substances

to be tested. The vials had closed lids, but evaporation occurred through two holes (0.5 mm diam.) just below the lid. We used one vial per substance, i.e., four vials, each with one of the four component odors, were attached to the trap. The traps were emptied each week (i.e., four times), and the substances were renewed once in 1994 (July 6) and every fortnight in 1997. When emptying the traps, we checked the baits to see that there were still substances left.

Throughout the study all insects trapped were collected and most beetles were identified to species. The literature used for the identification was the one listed in the paper by Jonsell and Nordlander (1995), and the nomenclature followed Silfverberg (1992). The attraction pattern was analyzed for beetles caught in numbers exceeding 15 individuals and for a moth caught in large numbers. All individuals of significantly attracted species were sexed. Statistical comparisons of catches with differently baited traps were made separately for each taxon identified. Catch numbers, x, were log $(x+1)$ transformed, to give a normal distribution and equal variances. Differences between the baits were analyzed with ANOVA, the model consisting of the bait-type and the block-number. Tukey's test was used for the multiple comparisons (Zar, 1984).

RESULTS

Major Odoriferous Volatiles. The volatiles recovered from the living fruiting bodies are presented in Figure 1 and Table 1. The main odor component of *F. pinicola* was a sesquiterpene. By evaluating the mass and NMR data obtained and comparing the results with corresponding data available in the literature (König et al., 1996), the compound was identified as β-barbatene [(3a.α.,4.α.,8.α.,8a.α.)-decahydro-3a,4,8a-trimethyl-7-methylene-4,8-methanoazulene] (Figure 2 and Table 2). The chirality of the compound has not been determined, nor have the complete assignments of the ^{1}H and ^{13}C NMR spectra been reported. For this reason, detailed analyses by means of the DQCOSY, NOESY, HSQC, and HMBC methods were conducted. The unambiguous identifications of the quaternary carbons by the 1,1-ADEQUATE experiment indicated a two-bond coherence transfer (C—C—H) between a methyl proton singlet and a quaternary carbon atom (Reif et al., 1996). The resulting assignments of the spectral data are summarized in Table 3.

Another main constituent was oct-1-en-3-ol (Table 1), both enantiomers of which were present. Other compounds released were, for example, octan-3-one, oct-1-ene and an isomer of β-barbatene (abbreviated ST B in Table 2). Benzyl alcohol was the major component in one sporulating fruiting body (Figure 3), but otherwise it was absent or released in small amounts.

The main volatiles released from the living fruiting bodies of *Fomes fomentarius* were octan-3-one, (−)-β-phellandrene, (2*S*, 5*R*)-*cis*-furanoid linalool

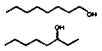
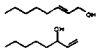
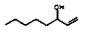
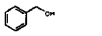
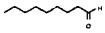
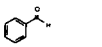
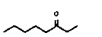
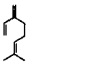
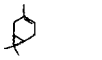
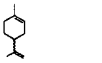
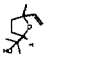
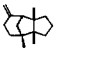
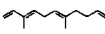
Compound	Structure	S	*Fomitopsis pinicola*		*Fomes fomentarius*	
			in situ	Chopped in week 1	*in situ*	Chopped in week 1
oct-1-ene		S	*	**	tr	*
octan-1-ol		S	*	*		*
octan-3-ol		S	*	*		*
oct-2-en-1-ol			*	*		
oct-1-en-3-ol		S	**	**		**
benzyl alcohol		S	tr			
nonanal			tr	tr	*	*
benzaldehyde		S	tr	tr	*	**
octan-3-one		S	**	**	**	**
α-pinene			*	*		*
β-myrcene		S			*	
3-carene			*	tr		
limonene			*	*	*	*
β-phellandrene		S			**	*
furanoid linalool oxide (5-ethenyltetrahydro-α, α,5-trimethyl-2-furanmethanol)		S			**	
4,5-dihydro-6-ethenyl-2,2,6-trimethyl-3-pyranone		S			*	
β-barbatene		S	**	**	tr	*
farnesene			*		*	
sesquiterpene fraction			**	***	***	***

FIG. 1. Compounds emitted by *Fomitopsis pinicola* and *Fomes fomentarius*; tr = trace, * = <6%, ** = 6–20%, *** = >21%. S = sporulation. Compounds emitted from the fruiting bodies in situ during sporulation. The sesquiterpene fraction denotes a gas chromatographic fraction containing sesquiterpene hydrocarbons only.

TABLE 1. RELATIVE AMOUNTS OF VOLATILES EMITTED BY POLYPORE FRUITING BODIES *In Situ*[a]

Polypore species/ compound name	Average amount (%)	Highest amount (%)	Frequency of occurrence (%)
Fomitopsis pinicola			
β-Barbatene	12	74	55
Oct-1-en-3-ol	5.3	27	55
Octan-3-one	4.0	19	50
Oct-1-ene	1.3	6.8	30
Limonene	1.0	7.2	30
Octan-1-ol	0.9	7.6	25
ST B[b]	0.9	4.6	30
α-Pinene	0.6	5.7	20
Farnesene	0.5	4.0	20
Octan-1-ol	0.5	3.0	25
Oct-2-en-1-ol	0.5	4.0	15
Fomes fomentarius			
ST fraction[c]	23	83	83
β-Barbatene	0.5	2.0	33
β-Phellandrene	14	43	33
cis-Furanoid linalool oxide	8.5	26	67
Octan-3-one	6.1	25	67
β-Myrcene	4.2	16	50
Nonanal	2.6	12	50
Benzaldehyde	1.2	4.0	50
4,5-Dihydro-6-ethenyl-2,2,6-trimethyl-3-pyranone	1.7	8.5	33

[a]Dynamic headspace samples collected from six fruiting bodies of *Fomitopsis pinicola* (20 samples) and six of *Fomes fomentarius* (six samples).
[b]The MS fragmentations of unknown sesquiterpene hydrocarbons are listed in Table 2.
[c]A gas chromatographic fraction, containing only sesquiterpene hydrocarbons. The relative amounts are based on the sums of the peak areas of the sesquiterpene hydrocarbons.

oxide [(2*S*, 5*R*)-*cis*-ethenyltetrahydro-α, α,5-trimethyl-2-furanmethanol], and β-myrcene (Figure 1 and Table 1). Nonanal, benzaldehyde, and a large number of unidentified sesquiterpene hydrocarbons (more than 11) were also emitted (Figure 1 and Table 2). The predominant compound within the group of sesquiterpenes in *F. pinicola*, β-barbatene, was a minor constituent in a few *F. fomentarius* samples. The enantiomeric compositions of the compounds analyzed were as follows: (−)-β-phellandrene >99% (one sample analyzed); (−)-limonene 85% (one sample analyzed); (−)-*cis*-furanoid linalool oxide >99% [(2*S*, 5*R*)-*cis*-furanoid linalool oxide, two samples analyzed]. Additional compounds released from *F. fomentarius* and collected by means of the SPME techniques were ethanol and acetic acid.

Seasonal Variation. As early as after four days at room temperature, three

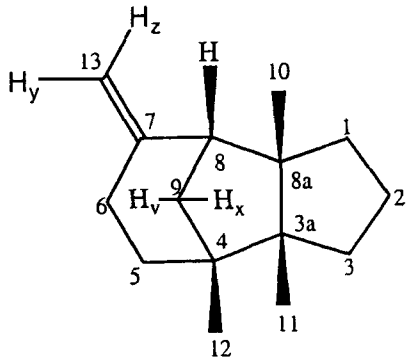


FIG. 2. Structure of β-barbatene.

of the *F. pinicola* fruiting bodies brought indoors started to sporulate, grow, and develop droplets of water. The activity continued for approximately one month, but the patterns of watering and sporulation differed among the individuals. The *F. fomentarius* specimens did not respond so readily, but after five to six weeks two fruiting bodies started to grow and lightly sporulate.

The odor patterns of *F. pinicola* in situ and of the samples taken from the specimens brought indoors were the same. During sporulation, there was an obvious increase in emission of octan-3-one, oct-1-en-3-ol, octan-3-ol, benzyl alcohol, and β-barbatene (Figure 3). Other compounds found during sporulation are labeled with the letter S in Figure 1.

The odor composition was altered in both species during the vegetation period in the field in situ (May to October). In the phases of inactivity, the con-

TABLE 2. MASS SPECTRAL DATA OF SESQUITERPENE HYDROCARBONS ST A–ST E[a]

Designation	Molecular fragments (%)
ST A (β-barbatene)	204 $[M]^+$ (4), 111 (36), 109 (27), 108 (100), 96 (90), 95 (60), 94 (63), 93 (66), 81 (41), 79 (22)
ST B	204 $[M]^+$ (38), 123 (25), 109 (27), 108 (100), 107 (42), 106 (22), 96 (42), 95 (53), 94 (43), 93 (78)
ST C (farnesene)	204 $[M]^+$ (11), 161 (38), 133 (53), 120 (43), 93 (76), 91 (21), 81 (22), 79 (25), 69 (100), 41 (46)
ST D	204 $[M]^+$ (17), 162 (12), 161 (100), 160 (5), 133 (9), 120 (15), 119 (16), 105 (23), 93 (8), 91 (15)
ST E	204 $[M]^+$ (39), 189 (22), 176 (16), 175 (100), 161 (13), 133 (24), 119 (52), 107 (11), 105 (26), 91 (12)

[a]As designated in this table and in Table 4.

TABLE 3. ^{13}C (125 MHz) AND ^{1}H (500 MHz) NMR SPECTRAL DATA OF β-BARBATENE IN $CDCl_3$ AT 25°C

C No.[a]	δC (ppm)[b]	δ_H (ppm)[c]	Multiplet[d]	Coupling constants (J_{HH}, Hz)
1	37.0	α 1.73	m	
		β 1.16	m	
2	27.5	α 1.78	m	
		β 1.78	m	
3	35.5	α 1.90	m	
		β 1.08	m	
3a	54.1			
4	43.0			
5	38.0	α 1.68	ddd	13.7, 8.5, 2.9
		β 1.39	ddd	13.7, 12.1, 8.1
6	28.7	α 2.43	dddt	16.4, 12.1, 8.5, 2.7
		β 2.20	dd	16.4, 8.1
7	152.0			
8	56.0	2.13	d	5.0
8a	55.4			
9	46.8	v 1.28	d	11.3
		x 2.03	ddd	11.3, 5.0, 2.9
10	27.5	1.01	s	
11	23.3	0.88	d	~0.4
12	24.8	0.82	s	
13	107.5	y 4.58	td	2.7, 0.7
		z 4.55	br. t	2.7

[a]The carbon atoms as numbered in Figure 2.
[b]The chemical shifts of the ^{13}C NMR spectra.
[c]The chemical shifts of the ^{1}H NMR spectra.
[d]The coupling pattern: s = singlet, d = doublet, t = triplet, m = multiplet, br = branched.

tents of C_8 alcohols and octan-3-one were low, and only a few sesquiterpenes were found in small amounts in the volatile fraction (Figure 4). The low release rate in June was most probably an effect of low humidity and high outdoor temperature, with a daily maximum about 25°C.

Fruiting bodies of *F. fomentarius* that were obviously sporulating in the field (in situ) emitted larger amounts of β-phellandrene, *cis*-furanoid linalool oxide, β-myrcene, and octan-3-one than of the sesquiterpene fraction. Analysis of SPME-sampled volatiles showed the presence of octan-3-one from the two sporulating *F. fomentarius* fruiting bodies brought indoors. A few sesquiterpenes were present during the time of no sporulation.

Chopped Fruiting Bodies. Chopping of the fruiting bodies altered the bouquet of the bracket fungi. In *F. pinicola* the changes were restricted to the relative amounts of the components (Figure 1). The change was moderate on the

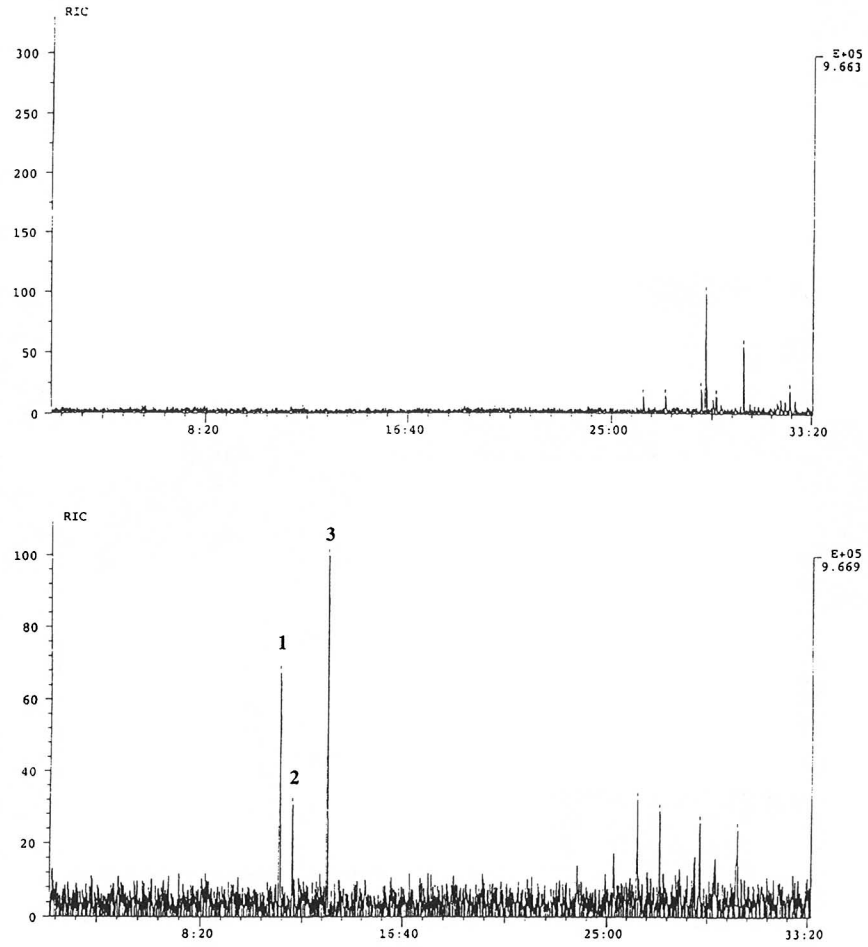


FIG. 3. Gas chromatograms of volatiles from *Fomitopsis pinicola* fruiting bodies sampled by the SPME technique, before sporulation (upper curve) and during sporulation (lower curve). Peak 1: octan-3-one, peak 2: octan-3-ol, peak 3: benzyl alcohol. The *y* axes denote the relative ion counts (RIC), and the total ion counts (TIC) are shown to the upper right. The *x* axes give the retention time in minutes and seconds.

first day after chopping. After one week, however, only small amounts of the C_8 compounds (i.e., oct-1-ene, oct-1-en-3-ol, octan-3-one, octan-1-ol, octan-3-ol, and oct-2-en-1-ol) remained, while the sesquiterpenes were the main constituents (Table 4). This pattern was even more pronounced after two weeks. The former group had almost disappeared from the bouquet (Figure 5).

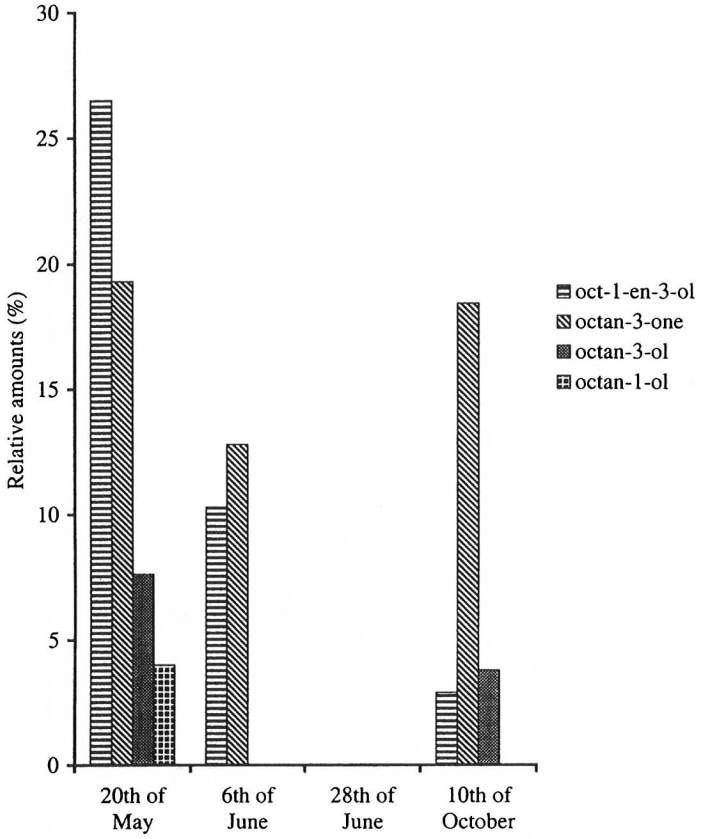


FIG. 4. Seasonal variation of volatiles emitted by one individual of *Fomitopsis pinicola*.

In contrast, chopping of the fruiting bodies of *F. fomentarius* caused a considerable qualitative change in the composition of the volatiles. Oct-1-en-3-ol was not detected in samples from *F. fomentarius* in situ, but it was an important constituent emitted from chopped fruiting bodies during the first days. Moreover, the main components in sporulating *F. fomentarius*, i.e., β-phellandrene, *cis*-furanoid linalool oxide, and β-myrcene, were not found in the odor of chopped fruiting bodies. Chopped *F. fomentarius* released about 20 sesquiterpenes constituting up to 91% of the total content of volatiles, while chopped *F. pinicola* released β-barbatene as a main component (up to 63%) and only a few additional sesquiterpenes (Table 4).

The multivariate data analysis provided another way of comparing the odor compositions. The score plot (Figure 6) shows the patterns of odor composi-

TABLE 4. RELATIVE AMOUNTS OF VOLATILES EMITTED BY CHOPPED FRUITING BODIES OF *Fomitopsis pinicola* AND *Fomes fomentarius*

	d1 + d7				d7 + d14			
	Average[a]	Highest	Lowest	f (%)[b] (N = 10)[c]	Average[a]	Highest	Lowest	f (%)[b] (N = 8)[c]
F. pinicola								
ST fraction[d]	30	93	0.8	100	57	93	14	100
β-Barbatene	15	63	0	90	24	63	6	100
ST B[e]	1.7	5.6	0	80	2.6	6.8	0	75
Farnesene	0.4	1.5	0	70	0.9	4.4	0	75
Oct-1-ene	18	63	0.4	100	2.3	13	0	50
Oct-1-en-3-ol	14	51	0	90	0.3	1.4	0	38
Octan-3-one	13	65	0.2	100	0.5	1.7	0	50
Octan-3-ol	4.1	14	0	80	0.6	1.7	0	38
Octan-1-ol	0.5	1.6	0	60	0	0	0	0
Oct-2-en-1-ol	0.2	0.8	0	40	0	0	0	0
F. fomentarius								
ST fraction[d]	46	91	8.1	70	41	91	12	75
ST D[e]	14	46	0	60	17	46	3.8	75
ST E[e]	7.9	14	0	60	4.5	11.3	0	63
β-Barbatene	4.2	17	0	40	0.2	1.4	0	25
Octan-3-one	18	42	0.7	70	2.7	7.2	0	63
Oct-1-en-3-ol	11	32	0	50	0.7	3.7	0	38
Benzaldehyde	2.8	11	0	60	2.3	5.2	0	50
Unknown	2.7	6.7	0	60	3.3	9.3	0	63
Nonanal	0.8	1.6	0	60	1	1.6	0	63

[a]Average values are given for days 1 + 7 and for days 7 + 14, representing weeks 1 and 2, respectively.
[b]The f values denote the frequencies of occurrence of the compounds in the samples.
[c]N = numbers of samples.
[d]A gas chromatographic fraction, containing only sesquiterpene hydrocarbons. The relative amounts are based on the sum of peakareas of sesquiterpene hydrocarbons.
[e]The MS fragmentation for unknown sesquiterpene hydrocarbons are listed in Table 2.

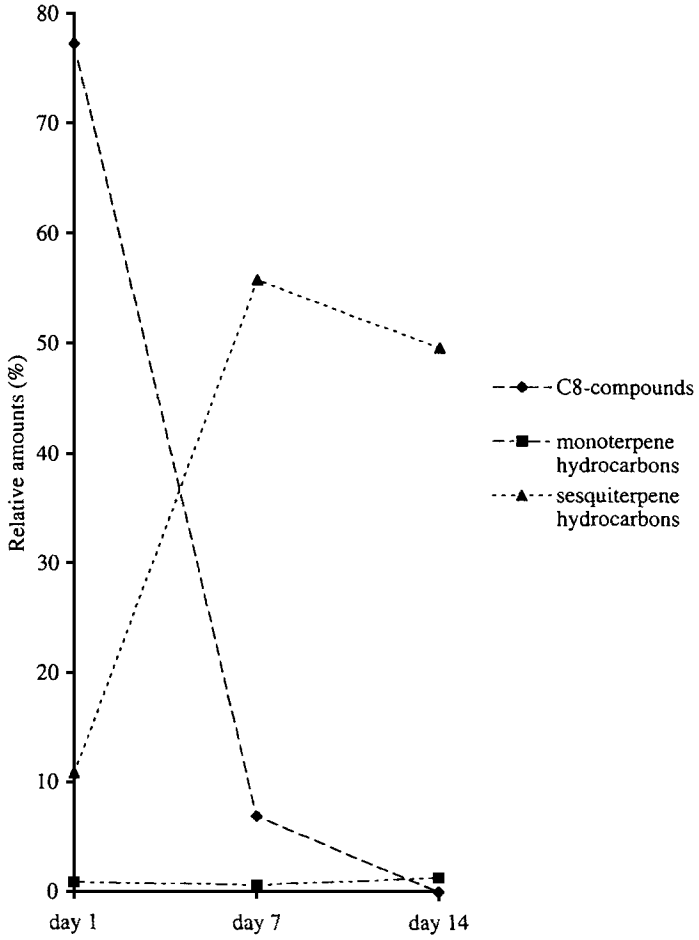


FIG. 5. Relative amounts of volatiles emitted by chopped *Fomitopsis pinicola* over time. Samples were taken on days 1, 7, and 14 after chopping. The C_8 compounds are oct-1-ene, oct-1-en-3-ol, octan-3-one, octan-1-ol, octan-3-ol, and oct-2-en-1-ol.

tion caused by the chopping and by the time that had elapsed after chopping of fruiting bodies. Samples of *F. pinicola* and *F. fomentarius* taken one day after chopping were rather similar, but differed from the corresponding samples taken after 7 and 14 days. The C_8 compounds were important for the position of the two groups containing samples taken one day after chopping of the both species.

The PLS-DA analysis showed a clear separation of in situ samples from *F. fomentarius* and samples taken at different times after chopping (Figure 7A

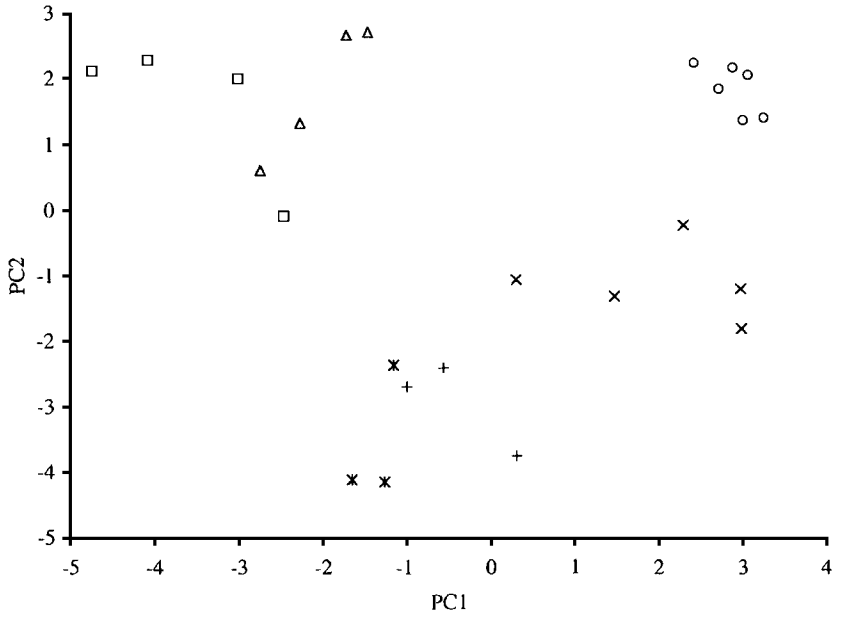
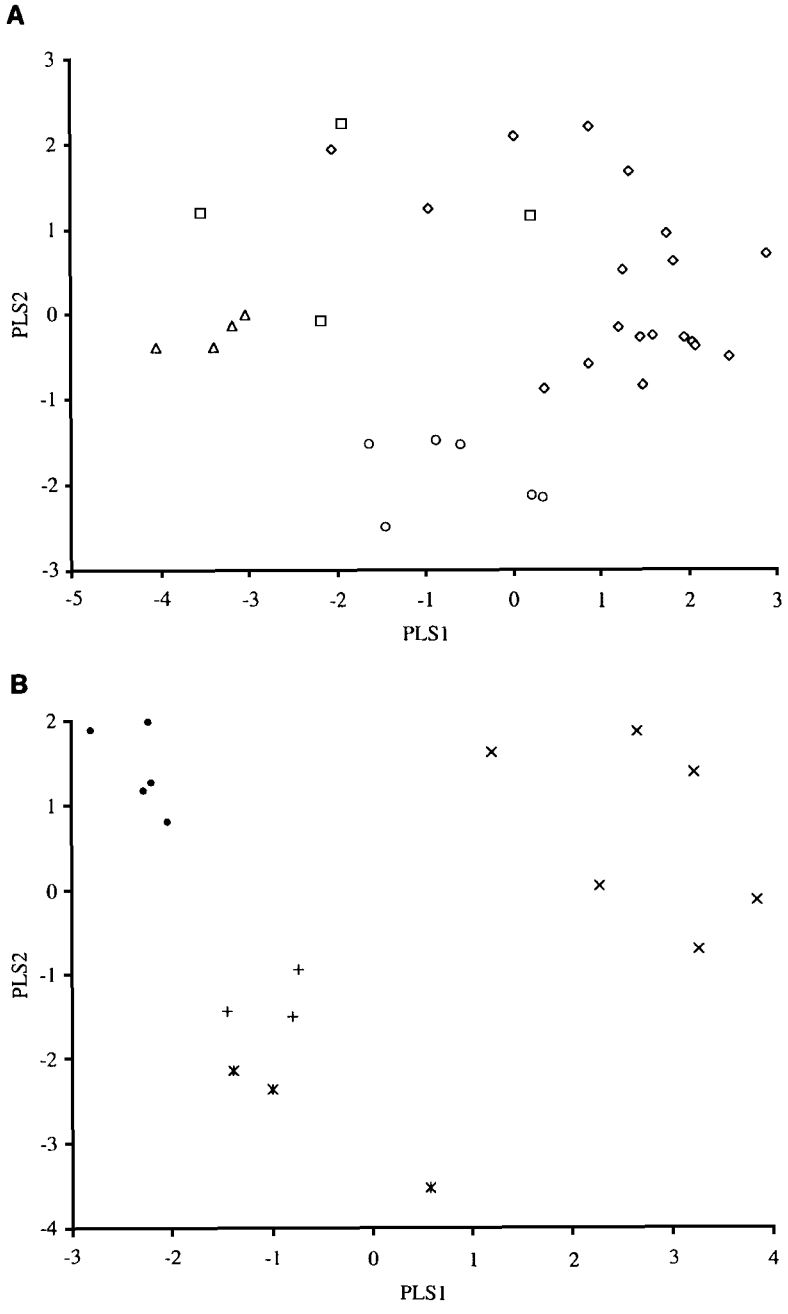


FIG. 6. Score plots from principal components analyses (PCA) based on total peak area obtained from GC-MS-data of headspace sorption samples (entrainment technique) from chopped fruiting bodies: *Fomitopsis pinicola:* days 1 (○), 7 (△), and 14 (□). *Fomes fomentarius:* days 1 (×), 7 (∗), and 14 (+). Both components were significant as judged by cross-validation, and they explained in total 33% of the variation in the GC data.

and B). The separation was less pronounced in *F. pinicola*, with a considerable overlap between samples from fruiting bodies in situ and samples taken 14 days after chopping. Compounds important for grouping samples from *F. fomentarius* in situ were β-phellandrene, *cis*-furanoid linalool oxide, and β-myrcene, but the absence of the C_8 alcohols was also typical.

FIG. 7. Score plots from projections to latent structures discriminant analysis (PLS-DA) based on total peak area obtained from GC data of headspace sorption samples (entrainment technique) from: (A) *Fomitopsis pinicola* in situ (◇) and chopped fruiting bodies of *F. pinicola*, on days 1 (○), 7 (△), and 14 (□). The first and only significant principal component, as judged by cross-validation, explains 13% of the variance in the GC data and 55% of the variance of the dummy variable. (B) *Fomes fomentarius* in situ (●) and chopped fruiting bodies of *F. fomentarius* on days 1 (×), 7 (∗), and 14 (+). The first and only significant principal component, as judged by cross validation, explains 14% of the variance in the GC data and 75% of the variance of the dummy variable.


A
PLS2
PLS1
B
PLS2
PLS1

Insect Attraction. Thirty-two beetle taxa and one moth species were caught in numbers high enough for statistical analyses, i.e., more than 15 specimens. Two of the beetle species, *Anaspis marginicollis* Lindberg and *A. rufilabris* (Gyllenhal), as well as the moth *Epinotia tedella* (Clerk) were significantly attracted to oct-1-en-3-ol (Table 5). The beetle *Malthodes fuscus* (Waltl) was also attracted to oct-1-en-3-ol in the first experiment, but this was not verified in the second experiment. The addition of other components to the oct-1-en-3-ol bait neither increased the number of insects attracted nor attracted additional species, except for *Lordithon lunulatus* (L.), which was attracted to oct-1-en-3-ol and octan-3-one in combination. No species was attracted to octan-3-one alone.

There was a conspicuous difference in attraction between the sexes of the two *Anaspis* species. Females were strongly attracted to oct-1-en-3-ol baited traps ($P < 0.001$), whereas male catches were not significantly greater than in traps baited with controls (Table 6). In one experiment, however, significantly more *A. rufilabris* males were caught in the oct-1-en-3-ol/octan-3-one traps than in those with octan-3-one alone ($P = 0.023$, Tukey's-test). A similar difference in attraction between sexes was also recorded for *Malthodes fuscus* in the first experiment. In *L. lunulatus*, males and females seemed to be similarly attracted, although the numbers of individuals were too low to allow statistical testing. Both sexes of *E. tedella* were significantly attracted, even though the sex ratio was skewed (about 30% females).

DISCUSSION

In situ samples of odors are essential for studies of the ecological role of these volatiles as cues for insects using polypores as food resources or breeding substrates. To our knowledge, this is the first study describing the composition of volatiles emitted from polypore fruiting bodies in situ. Earlier studies have used mycelium cultures on synthetic media, with the aim of finding taxonomic characters or aroma products (Gallois et al., 1990). Many of the constituents that we found in *Fomitopsis pinicola* and *Fomes fomentarius* are known as general fungal volatiles, e.g., oct-1-en-3-ol and octan-3-one (Pyysalo, 1976; Kaminski et al., 1980; Hanssen et al., 1986; Gallois et al., 1990; Abraham and Berger, 1994). Mono- and sesquiterpenoids also have been found in other fungi (Turner and Aldridge, 1993), but the main constituent of the *F. pinicola* volatiles, β-barbatene, has not earlier been found in fungi, although it has been isolated and identified in liverworts (Hepatophyta) (Andersen et al., 1973; Matsuo et al., 1985) and in an Apiaceae species (König et al., 1996). The monoterpenes limonene and α-pinene, which are minor constituents in both species, are known to be main constituents in extracts from the xylem of *Picea abies* (Persson et al., 1996). In

TABLE 5. NUMBERS OF INDIVIDUALS CAUGHT IN DIFFERENTLY BAITED TRAPS IN TWO INSECT ATTRACTION EXPERIMENTS

Species	Experiment 1[a]				Experiment 2[b]				
	C	A	Mix	P[c]	C	A	K	A + K	P[c]
Acrotrichis Moutsch. spp.	–	–	–		4	4	5	4	
Agathidium seminulum (L.)	–	–	–		2	5	4	5	
Philonthus succicola Thoms.	–	–	–		7	8	6	1	
Lordithon lunulatus (L.)[d]	–	–	–		0	0	3	20	<0.001
Atheta spp. s.l.[d]	4	8	10		4	6	5	4	
Bibloporus bicolor (Denny)	–	–	–		10	13	13	10	
Euplectus Leach spp.[d]	–	–	–		7	8	4	7	
Rhagonycha Esch. spp.	11	13	16		5	5	8	6	
Malthinus Latr. spp.	8	8	16		8	10	12	7	
Malthodes brevicollis (Payk.)	20	28	24		9	4	6	7	
Malthodes fuscus (Waltl)	6	30	17	0.017	15	12	14	8	
Athous subfuscus (Müll.)	55	42	43		26	28	24	13	
Melanotus Esch. spp.	8	9	10		12	10	12	14	
Dalopius marginatus (L.)	4	7	7		3	9	4	4	
Dasytes plumbeus (Müll.)	11	18	11		3	10	12	1	
Cychramus variegatus (Hbst.)	–	–	–		0	14	0	7	
Sphindus dubius (Gyll.)	–	–	–		13	2	6	3	
Dendrophagus crenatus (Payk.)	6	4	6		3	3	5	7	
Cryptophagus abietis (Payk.)	–	–	–		7	7	5	4	
Atomaria Steph. spp.	19	18	18		30	30	29	24	
Cerylon histeroides (Fabr.)	–	–	–		6	6	5	3	
Cerylon ferrugineum (Steph.)[d]	8	6	5		5	8	4	2	
Enicmus rugosus (Hbst.)	13	15	16		64	56	54	68	
Enicmus testaceus (Steph.)[d]	12	4	8		60	105	98	89	
Salpingus ruficollis (L.)[d]	12	12	21		7	10	10	11	
Anaspis marginicollis Lindb.	–	–	–		3	27	4	19	<0.001
Anaspis rufilabris (Gyll.)[d]	21	77	89	0.001	23	65	13	91	<0.001
Xylita laevigata (Hell.)	8	8	9		–	–	–		
Hylastes cunicularius Er.	–	–	–		14	12	22	16	
Crypturgus spp.	116	112	62		41	26	27	22	
Dryocoetes spp.	14	9	6		16	6	10	16	
Epinotia tedella (Cl.)	37	335	378	<0.001	–	–	–		

[a]Baits in experiment 1: C, empty control; A, oct-1-en-3-ol; Mix, octan-1-ol, octan-3-ol, nonan-1-ol, and oct-1-en-3-ol.

[b]Baits in experiment 2: C, empty control; A, oct-1-en-3-ol; K, octan-3-one; A + K, oct-1-en-3-ol and octan-3-one.

[c]*P* values are included only in case of significant attraction to odor bait (ANOVA).

[d]Species that were attracted to odor of chopped polypores in a previous study (Jonsell and Nordlander, 1995).

TABLE 6. NUMBERS OF INDIVIDUALS OF EACH SEX OF SPECIES ATTRACTED[a] CAUGHT IN TRAPS[b]

	Experiment 1				Experiment 2				
Species and sex	C	A	Mix	P^c	C	A	K	A + K	P^c
Lordithon lunulatus									
Males	—	—	—		0	0	1	8	
Females	—	—	—		0	0	0	5	
Malthodes fuscus									
Males	4	11	4	0.083	4	4	6	2	0.57
Females	2	19	13	0.001	10	8	6	5	0.81
Anaspis marginicollis									
Males	—	—	—		2	3	1	4	0.76
Females	—	—	—		1	17	1	11	<0.001
Anaspis rufilabris									
Males	7	14	29	0.66	6	6	2	13	0.037
Females	12	60	56	<0.001	17	56	11	68	<0.001
Epinotia tedella									
Males	9	89	128	<0.001	—	—	—	—	
Females	3	39	56	0.004	—	—	—	—	

[a]A few were not sexed.
[b]Abbreviations as in Table 5.
[c]*P* values indicate statistical probabilities of differences in numbers between baits of the same kind (ANOVA).

the main host of *F. fomentarius*, i.e., *Betula* spp., only traces of monoterpenes have been found in the xylem (Borg-Karlson, unpublished).

One of the compounds tested in the field, the general fungal volatile oct-1-en-3-ol, attracted some saproxylic beetles with comparatively broad substrate requirements. When presented alone, oct-1-en-3-ol attracted females of *Anaspis marginicollis*, *A. rufilabris*, and perhaps also *Malthodes fuscus*, all of which breed in various kinds of rotten wood. In combination with oct-1-en-3-ol, octan-3-one attracted *L. lunulatus*, a predatory species found on various mushrooms and polypores (Palm, 1959; Hansen, 1964). Only females of the wood-living species *A. marginicollis*, *A. rufilabris*, and *M. fuscus* were attracted to the odor baits. This suggests that the males are attracted to the breeding substrate by a female-produced pheromone as is the case with *Dorcatoma robusta*, breeding in *F. fomentarius* (Jonsson et al., 1997). No difference in attraction between sexes was observed for *L. lunulatus*, in which the adults act as predators on fungivorous insects. Saproxylic beetles with more narrow substrate requirements were not attracted to the tested compounds, although 16 species in this category were attracted to odor baits of chopped fruiting bodies (Jonsell and Nordlander, 1995). The number of fungal substrates releasing the aliphatic compounds we tested is

probably too large to be useful for these insects as the sole cues for locating suitable breeding substrates.

Volatiles from the two polypore species studied attract beetles breeding in polypores and in decaying wood (Jonsell and Nordlander, 1995). Furthermore, the two cisid beetles *Cis glabratus* Mellié and *C. quadridens* Mellié can discriminate the host odor of chopped fruiting bodies of *F. pinicola* from the odor of chopped *F. fomentarius* (Jonsell and Nordlander, 1995). The odor compositions of the two chopped fungi were rather well separated in the PCA plot. However, the C_8 compounds from the odor of *F. pinicola* presented in the field experiments did not reveal any attraction of cisids. Most likely, these beetles use species-specific differences in the sesquiterpene pattern when locating new patches. One substance that might be used is β-barbatene, which was present in a high proportion in *F. pinicola*, while it was only a minor constituent in *F. fomentarius*.

Jonsell and Nordlander (1995) did not find any beetle monophagous on *F. fomentarius* to be attracted by the odor of their host, whereas some species associated with *F. pinicola* were attracted to their appropriate host-odor baits, which can be explained by the fact that their odor-baits consisted of chopped fruiting bodies. When comparing chopped and living fruiting bodies, a larger difference in odor composition was found for *F. fomentarius* than for *F. pinicola*. In both species the effect of chopping seems to be more important the first day than after one or two weeks. Many volatile compounds are rapidly released just after the chopping, and when they are evaporated, the odor composition is stabilized nearer the starting point, i.e., the composition in a living fruiting body. The baits used by Jonsell and Nordlander (1995) were operated for two weeks and thus the insects were mainly exposed to the stabilized composition. Even when the samples taken the first day are omitted, chopping alters the odor pattern more in *F. fomentarius* than in *F. pinicola*. The important changes in *F. fomentarius* odor after chopping were the increase of oct-1-en-3-ol and the absence of monoterpenoids such as *cis*-furanoid linalool oxide, β-phellandrene, and β-myrcene.

The presence of certain degradation products may also be of importance to the insects. Most of the species breeding in these polypores oviposit in dead or dying fruiting bodies (Matthewman and Pielou, 1971; and personal observation). Volatiles of dead or dying biological substrates are often characterized by degradation products such as ethanol or other oxidation products, which may play a significant role in insect attraction during the dying phase. Ethanol and acetic acid were detected from certain individuals of *F. fomentarius* brackets, which spread an acetic acid-like smell when volatiles were sampled with the SPME device. Thus, only a few of the *F. fomentarius* fruiting bodies may actually be attractive to associated insects.

Larger amounts of volatiles were released during sporulation than during inactive phases. These volatiles might act as signals for resource location by insects feeding on spores. Many insects that develop under the bark of logs are

regularly found on the hymenium of fruiting bodies, where they feed on spores. One example is *Pteryngium crenatum* (F.), which is attracted to the odors of both *F. pinicola* and *F. fomentarius* (Jonsell and Nordlander, 1995). Polypores show a highly variable rate of spore release over the season, depending on weather and species-specific rhythms (Nuss, 1975; Soukup, 1987). If insects are important for spore dispersal (Harrington et al., 1981), the increased amounts of volatiles could be adaptive for the fungus by attracting insect vectors.

The moth *Epinotia tedella*, which feeds on spruce needles (*Picea abies*) was strongly attracted to racemic oct-1-en-3-ol. This was not expected as this compound is neither known to be emitted from fresh needles (Persson et al., 1996) nor to be a pheromone compound (Priesner et al., 1989; Roelofs and Brown, 1982). A sex pheromone function is further rejected as both sexes were attracted. However, *E. tedella* larvae prefer needles that are at least 2 years old (Hansen, 1987; Münster-Swendsen, 1989), and such needles are usually colonized by endophytic fungi, mainly *Lophodermium piceae* Fuckel (Höhn.) (Barklund, 1987). The chemically related octan-3-on has been identified in volatiles from 2-year-old spruce needles infected by *L. piceae* (Fäldt et al., unpublished data). If the endophytic fungi enhance the food quality of the needles, oct-1-en-3-ol could, at a certain developing state, be released and act as a cue for locating the preferred needles. However, earlier studies have only found that endophytic fungi may be negative to herbivores (Bernays and Chapman, 1994), e.g., to the spruce budworm feeding on conifer needles (Clark et al., 1989).

Volatiles emitted from fungi are certainly important to associated insects. There are large differences in volatile composition both among fungi of different species and within species. Both the death of a fruiting body and the onset of sporulation significantly alter the quality and quantity of the odor bouquet. Such changes are probably extensively used by insects in their search for new patches suitable for their reproduction.

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SPECIFICITY OF RESPONSE TO SEX PHEROMONES AMONG SWEETPOTATO WEEVILS, *Cylas puncticollis* AND *C. brunneus*

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Abstract—Trapping experiments were carried out in Uganda and Indonesia with decyl (*E*)-2-butenoate and dodecyl (*E*)-2-butenoate, previously identified as components of the female sex pheromones of the African species of sweetpotato weevil, *Cylas puncticollis* and *C. brunneus*, respectively. In Uganda, decyl (*E*)-2-butenoate attracted only *C. puncticollis* males, but dodecyl (*E*)-2-butenoate attracted males of both *C. brunneus* and *C. puncticollis*. Catches of *C. puncticollis* with both compounds were higher when they were dispensed from polyethylene vials rather than rubber septa, while dodecyl (*E*)-2-butenoate dispensed from rubber septa gave higher catches of *C. brunneus* and was more selective for this species. Release rates of the two compounds from the two types of dispenser were measured in the laboratory and possible explanations for differences in dispenser performance considered. Lures containing decyl (*E*)-2-butenoate were as attactive to male *C. puncticollis* as 10 live virgin female *C. puncticollis* weevils, but lures containing dodecyl (*E*)-2-butenoate were not always as attractive to male *C. brunneus* as the conspecific virgin female weevils. Dose–response relationships with the synthetic pheromones varied between repeat experiments and with the type of dispenser. Addition of dodecyl (*E*)-2-butenoate to decyl (*E*)-2-butenoate either did not affect or increased catches of *C. puncticollis* males, but adding 1% or more of decyl (*E*)-2-butenoate to dodecyl (*E*)-2-butenoate significantly reduced attrac-

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tiveness to *C. brunneus*. Traps baited with synthetic lures captured male *C. brunneus* weevils mostly early in the evening while the majority of male *C. puncticollis* were trapped between 01:00 hr and 03:00 hr. This clear temporal separation of activity of males of the two species helps to ensure species specificity of mating in these sympatric species. In Indonesia, dodecyl (*E*)-2-butenoate but not decyl (*E*)-2-butenoate attracted *C. formicarius* males, but this attractiveness was less than 0.4% that of the pheromone of *C. formicarius*, (*Z*)-3-dodecenyl (*E*)-2-butenoate.

Key Words—Sweetpotato weevils, *Cylas puncticollis*, *Cylas brunneus*, *Cylas formicarius*, pheromone trap, *Ipomoea batatas*, Uganda, Indonesia.

INTRODUCTION

The most important insect pests of sweetpotato, *Ipomoea batatas* L., worldwide are sweetpotato weevils of the genus *Cylas* (Coleoptera: Apionidae) (Jansson and Raman, 1991). Damage results from larval feeding within tubers, and even at low infestation rates the plants produce unpalatable terpenoids in response to this (Uritani et al., 1975). The cryptic feeding habits of the larvae and the nocturnal activity of the adults make detection and control of infestations difficult.

Cylas formicarius F. is the most widespread species of sweetpotato weevil, and the female-produced sex pheromone was identified as (*Z*)-3-dodecenyl (*E*)-2-butenoate and synthesized by Heath et al. (1986). Use of this pheromone in pest management has been investigated in the United States, the Caribbean, and Southeast Asia (Jansson et al., 1991). Mass trapping of males has been shown to suppress populations of *C. formicarius* males in Taiwan (Hwang and Hung, 1991), India (Pillai et al., 1993), Vietnam (Braun and van de Fliert, 1997), Indonesia (Braun and van de Fliert, 1999) and Japan (Yasuda, 1995). Use of the pheromone in mating disruption of *C. formicarius* has also been reported (Mason and Jansson, 1991).

In Africa the most important species are *C. puncticollis* Boheman and *C. brunneus* F. (Wolfe, 1991; CAB International, 1993), which are restricted to Africa. The females of both species produce a sex pheromone that attracts conspecific males (Smit et al., 1994; Sathula, 1993), and Hall et al. (unpublished data) identified the major component of the pheromone of *C. puncticollis* as decyl (*E*)-2-butenoate and that of *C. brunneus* as dodecyl (*E*)-2-butenoate. Initial trapping experiments with the synthetic pheromones (Smit et al., 1997; Hall et al. (unpublished data), showed that decyl (*E*)-2-butenoate attracts only *C. puncticollis* males but dodecyl (*E*)-2-butenoate attracts males of both *C. brunneus* and *C. puncticollis*, suggesting the identifications might be incomplete.

This paper reports further field experiments in Uganda to confirm the pheromone identifications and explain how specificity of mating of the two weevil species is ensured. This work is important for development of trapping and

control programs employing the respective pheromones (Laboke et al., 1997). An experiment carried out in Indonesia to investigate the possible attractiveness of the *C. puncticollis* and *C. brunneus* pheromones for *C. formicarius* is also described. Although *C. formicarius* does not generally coexist with the other two species in Africa, it has been noted at two coastal locations in Kenya and South Africa (Wolfe, 1991; Parker et al., 1992), probably as an accidental import, and is similar in appearance to *C. brunneus*.

METHODS AND MATERIALS

Experiments in Uganda. With the exception of an experiment with *C. formicarius*, the trapping experiments were carried out in Uganda between February 1995 and June 1997 in sweetpotato fields at the Namulonge Agricultural and Animal Production Research Institute (NAARI) (0°32′N, 32°35′E, 1128 m above sea level, mean annual rainfall 1270 mm spread over two seasons). Traps were placed in sweetpotato fields of 0.3–0.5 ha planted with several different varieties. The fields were either being used for unrelated experiments and had natural weevil infestations or were specifically planted for the trapping experiments and artificially infested with weevils of both species. Crops were rain-fed and cultural practices were similar in all fields with no herbicides, fungicides, insecticides, or fertilizers used.

Pheromones and Lures. Decyl (*E*)-2-butenoate and dodecyl (*E*)-2-butenoate were synthesized at NRI as described by Hall et al. (unpublished data) and were at least 98% pure by gas chromatographic (GC) analysis. Pheromone dispensers were polyethylene vials (23 mm × 9 mm × 1.5 mm thick; Just Plastics, London E10 7PY, U.K.) or white rubber septa (part No. Z10,072-2; Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K.). These were impregnated by adding the pheromone dissolved in hexane (0.1 ml) containing an equal weight of 2,6-di-*tert*-butyl-4-methylphenol (BHT) as antioxidant, and allowing the solvent to evaporate. The polythene vials were capped after loading the pheromone.

In trapping experiments with female weevils as bait, five or ten 2-week-old virgin females of the appropriate species and some sweetpotato tuber were placed in metal wire mesh cages which were suspended in the traps (Smit et al., 1997). Preliminary experiments showed that the presence of the cage did not adversely affect catches. The females and tuber piece were checked and replaced as appropriate every three days. Controls consisted of an identical cage containing only a piece of tuber.

Traps. Three trap types were used: the skirted funnel trap, the 5-liter polythene jerry-can trap, and the 1-liter bottle trap, all described by Smit et al. (1997). The study by Smit et al. (1997) found that catches of *C. puncticollis* and *C. brunneus* were highest with the 1-liter bottle and the funnel designs, respec-

tively. However, the jerry-can trap caught large numbers of both species and was much more practical than either of the other two designs. The trapping agent was a dilute solution of washing detergent and traps were positioned on field ridges. For experiments carried out after September 1995, the traps were raised on bricks as this was shown to increase catches of *C. puncticollis*, while having no effect on catches of *C. brunneus* (Smit et al., 1997). Separate traps were used for each target species. Initial experiments confirmed that all weevils trapped of both species were males, as determined by antennal morphology.

Design and Analysis of Trapping Experiments. Four or five treatments were compared within each experiment. Each was replicated four times. With the exception of the time-of-capture observations, experiments were initially laid out to a randomized complete-block design and traps were moved round one position on successive sample dates so that each treatment was present at each position, within each replicate, on one occasion in each experiment. Traps were separated by 10–25 m within replicates and replicates were separated by at least 50 m, typically with one field comprising one replicate. The numbers of males of each species caught per trap were recorded daily in the morning, unless otherwise indicated.

Individual trap-catch data were transformed to $\log_{10}(x + 1)$ values to stabilize error variances, then subjected to analysis of variance by using the factors of night of catch, replicate block, and treatment. In some analyses, consistent zero catches by one or more treatments resulted in heterogeneity among the variances even after data were transformed. In these cases the appropriate treatments were omitted from the final analysis. When ANOVA indicated that significant effects existed, comparisons among treatment means were made by calculating the appropriate least significant difference from the standard error of the difference and the appropriate value of t, obtained from tables.

Time-of-Capture Observations. Observations on the diel timing of catches of male weevils were made with jerry-can traps and 1-liter bottle traps. Dispensers used were septa containing 0.1 mg dodecyl (*E*)-2-butenoate for *C. brunneus* and vials containing 0.1 mg decyl (*E*)-2-butenoate for *C. puncticollis*. Nine separate sets of observations were carried out between April 1995 and August 1996 (Table 1). In each case there were four replicate traps. Sets 1 and 6 consisted of five separate morning and evening counts of catches. For other sets, catches were counted at intervals of an hour or more during each of four successive day or night periods. For sets 4, 5, 8, and 9, catches on the first two nights were counted at intervals of 2–5 hr, while on the third and fourth nights they were counted at hourly intervals over shorter periods. Sunrise and sunset were at approximately 07:00 and 19:00 hr, respectively.

Data are presented as the percentage of the total catch within an observation period that occurred during each trapping interval. For this purpose catches by all four traps were combined and the means and standard errors for all appropriate

TABLE 1. OBSERVATIONS ON TIME OF CAPTURE OF SWEETPOTATO WEEVILS

Species and observation	Type of trap	Periods of trapping		Times/intervals of trap counts
		Timing	*N*	
C. brunneus				
1	5-liter jerry-can	24 hr	5	08:00 and 17:00
2	1-liter bottle	16:00–09:00	4	3–5 hr
3	1-liter bottle	16:00–21:00	4	1 hr
4	1-liter bottle	09:00–21:00	2	2 hr
		16:00–21:00	2	1 hr
5	5-liter jerry-can	09:00–21:00	2	2 hr
		16:00–21:00	2	1 hr
C. puncticollis				
6	5-liter jerry-can	24 hr	5	08:00 and 17:00
7	1-liter bottle	16:00–09:00	4	3–5 hr
8	1-liter bottle	19:00–09:00	2	2–5 hr
		0:00–04:00	2	1 hr
9	5-liter jerry-can	19:00–09:00	2	2–5 hr
		0:00–04:00	2	1 hr

observation periods were calculated. Results from more than one observation set were combined where the intervals between trap counts corresponded. Where the total catch for a complete observation period was below 20 individuals the data were omitted.

Measurement of Release Rates of Synthetic Pheromones from Dispensers. Release rates of both pheromones from both types of dispenser were determined by exposing the lures with 1 mg loading of pheromone in a wind tunnel maintained at 27°C and with a continuous 8 km/hr windspeed. Duplicate samples were removed at intervals up to 100 days and stored in sealed, glass vials at −20°C. The amount of pheromone remaining in the lures was determined by extracting the pheromone at room temperature overnight with hexane (5 ml) containing hexadecyl acetate (1 mg) as internal standard. The solution was analyzed by GC with a fused silica capillary column (25 m × 0.32 mm ID) coated with CP Wax 52 CB (chemically bonded Carbowax 20 M equivalent; Chrompack, U.K.). The oven temperature was held at 120°C for 2 min and then programmed at 6°C/min to 230°C. Samples were injected by autosampler (AS 200S) and data were captured and processed with EZChrom 6.5 data handling hardware and software.

Trapping of C. formicarius *in Indonesia.* This experiment was carried out in farmers' sweetpotato fields at Leuwiliang, West Java, Indonesia in early October 1997. At this location only *C. formicarius* are found. Water-pan traps were used, modified from the design of Talekar and Lee (1989) without a wire-framed

base. In the first part of the experiment, traps with rubber septa lures containing decyl or dodecyl (*E*)-2-butenoate (0.1 mg) or a control blank were set out in a randomized complete block design with three-fold replication. In the second part, a fourth trap baited with a rubber septum impregnated with (*Z*)-3-dodecenyl (*E*)-2-butenoate (0.1 mg), the pheromone of *C. formicarius* was added to each replicate group, and a fourth replicate of all four treatments was set up in a separate field. Fields forming replicate blocks were situated at least 100 m apart. Trap catches were counted and the traps moved round one position daily for three and four days in each part of the experiment, respectively.

RESULTS

Pheromone Dispenser Comparisons. Comparisons were made of catches with decyl (*E*)-2-butenoate or dodecyl (*E*)-2-butenoate dispensed from polyethylene vial or white rubber septa dispensers at 0.1 mg loading. The experiment was replicated in skirted funnel and jerry-can traps, and an unbaited control was included in the design in each case.

Both experiments showed that decyl (*E*)-2-butenoate attracted *C. puncticollis* essentially exclusively while dodecyl (*E*)-2-butenoate attracted significant numbers of both species (Table 2). In each experiment more male *C. puncti-*

TABLE 2. CATCHES OF SWEETPOTATO WEEVILS IN FUNNEL AND JERRY-CAN TRAPS BAITED WITH SYNTHETIC PHEROMONE (0.1 mg) IN DIFFERENT DISPENSERS[a]

Trap/compound	Dispenser	Mean catch/trap/night (± SE)[b]		Ratio target/ nontarget
		C. puncticollis	*C. brunneus*	
Funnel traps				
Decyl (*E*)-2-butenoate	vial	20.6 ± 4.5 a	0.3 ± 0.1 c	68.7
Decyl (*E*)-2-butenoate	septum	11.2 ± 2.5 b	0.1 ± 0.1 c	112.0
Dodecyl (*E*)-2-butenoate	vial	6.8 ± 1.6 c	3.4 ± 1.0 b	0.5
Dodecyl (*E*)-2-butenoate	septum	2.0 ± 0.6 d	22.1 ± 5.1 a	11.0
Control (unbiated)		0.0 ± 0.0 e	0.0 ± 0.0 c	
5-liter jerry-can traps				
Decyl (*E*)-2-butenoate	vial	17.8 ± 4.9 a	0.7 ± 0.3 c	25.4
Decyl (*E*)-2-butenoate	septum	13.8 ± 2.8 a	0.5 ± 0.3 c	27.6
Dodecyl (*E*)-2-butenoate	vial	8.5 ± 2.7 b	14.2 ± 3.2 b	1.7
Dodecyl (*E*)-2-butenoate	septum	5.2 ± 3.2 c	29.0 ± 5.0 a	5.6
Control (unbaited)		0.2 ± 0.1 d	0.2 ± 0.1 c	

[a]Four replicates; five nights; February 1995 and 1996, respectively.

[b]Means within a column group followed by a common letter were not significantly different, based on comparison of each mean with the nearest lower mean value ($P < 0.05$ for LSD following ANOVA of log-transformed catches).

collis weevils were caught in traps baited with decyl (*E*)-2-butenoate dispensed from polyethylene vials than in those baited with rubber septa. In contrast, significantly more *C. brunneus* males were trapped when the dodecyl (*E*)-2-butenoate was dispensed from rubber septa than from polyethylene vials in both experiments. Furthermore, catches of *C. puncticollis* with dodecyl (*E*)-2-butenoate were higher when this was dispensed from vials rather than septa, so catches with dodecyl (*E*)-2-butenoate dispensed from septa contained a much higher proportion of *C. brunneus* relative to *C. puncticollis* than catches when this compound was dispensed from vials.

Trapping with Females and Dose–Response Comparisons. Seven experiments were carried out to compare catches in jerry-can traps baited with virgin female weevils or with lures containing 0.01, 0.1, or 1 mg of the synthetic pheromone components dispensed from polyethylene vials or rubber septa.

In all three experiments with female *C. puncticollis* and lures containing decyl (*E*)-2-butenoate, the virgin females and the lures containing 0.1 or 1.0 mg of synthetic pheromone attracted similar numbers of *C. puncticollis* males (Table 3). In the experiment with septa dispensers, the three loadings of pheromone attracted equal numbers of males, while in both experiments with vial dispensers there was a consistent increase in catch with each increase in pheromone loading, although the catches of the two highest loadings did not differ significantly. Numbers of *C. brunneus* males attracted were negligible.

In all four experiments with *C. brunneus* females and lures containing dodecyl (*E*)-2-butenoate, traps baited with 10 virgin females tended to catch more *C. brunneus* males than those baited with any of the three loadings of synthetic pheromone. However, the difference in catches with females and with the highest catching synthetic lure was only significant in two cases (Table 4). In all experiments, the 0.01-mg loading attracted far fewer males than the two higher loadings, and catches were similar to those in unbaited controls. In the three experiments using septa dispensers, the 0.1- and 1.0-mg loadings were equally attractive, while only in the experiment with vial dispensers was the 1.0-mg dose significantly more attractive. There was a marked cross-attractiveness of dodecyl (*E*)-2-butenoate to *C. puncticollis* males, as observed previously, but the *C. brunneus* females were much more specific in attracting conspecific males.

Linear regressions of log-transformed catch against log-transformed loading were performed for each experiment for the three synthetic lures only. Most, although not all, of the regression coefficients were significantly greater than zero ($P < 0.05$, t test), indicating an overall significant positive dose–response (Tables 3 and 4). The highest values for *C. puncticollis* and *C. brunneus*, responding to their respective synthetic pheromones, were 0.30 and 0.49, respectively, which indicated increases in catches of 2.0- and 3.1-fold for each 10-fold increase in dose.

Experiments with Combinations of Pheromone Components. Catches with lures containing the synthetic pheromone of the target species, decyl (*E*)-2-

TABLE 3. CATCHES OF SWEETPOTATO WEEVILS IN 5-LITER JERRY-CAN TRAPS BAITED WITH VIRGIN FEMALE *C. puncticollis* WEEVILS OR DECYL (*E*)-2-BUTENOATE DISPENSED FROM POLYETHYLENE VIALS OR RUBBER SEPTA (5 REPLICATES; 5 NIGHTS)

Experiment/lure	Mean catch/trap/night (±SE)[a]	
	C. puncticollis	*C. brunneus*
February 1995		
Control (tuber piece)	0.3 ± 0.2 c	0
10 virgin females + tuber piece	10.3 ± 3.1 a	0
0.01-mg vial	2.7 ± 0.7 b	0
0.1-mg vial	9.3 ± 2.0 a	0.2 ± 0.1
1.0-mg vial	12.7 ± 2.1 a	0.4 ± 0.2
Slope, *P* for dose–response[b]	0.30a, <0.001	
April 1995		
Control (tuber piece)	2.8 ± 0.7 b	0.1 ± 0.1
10 virgin females + tuber piece	7.0 ± 2.0 a	0.1 ± 0.1
0.01-mg septum	8.0 ± 2.5 a	0.1 ± 0.1
0.1-mg septum	7.6 ± 2.6 a	0.1 ± 0.1
1.0-mg septum	7.6 ± 2.4 a	0
Slope, *P* for dose–response[b]	0.02b, 0.78	
May 1996		
Control (tuber piece)	1.2 ± 0.8 c	0.1 ± 0.1
10 virgin females + tuber piece	14.5 ± 3.4 a	0.2 ± 0.1
0.01-mg vial	6.4 ± 1.1 b	1.0 ± 0.4
0.1-mg vial	10.8 ± 2.0 a	0
1.0-mg vial	16.8 ± 2.7 a	0
Slope, *P* for dose–response[b]	0.21ab, <0.001	

[a]For *C. puncticollis*, means within a column group followed by a common letter were not significantly different ($P < 0.05$ for LSD following ANOVA of log-transformed catches).

[b]Value of the regression coefficient b, and the significance of its departure from zero, found from the linear regression of $\log_{10}$-transformed catch on $\log_{10}$ (dose) ($y = a + bx$) for three loadings of synthetic pheromone; coefficients followed by a common letter indicate they are not significantly different [$P < 0.05$, by method of Bailey (1981, p. 97)].

butenoate for *C. puncticollis* or dodecyl (*E*)-2-butenoate for *C. brunneus*, combined with varying proportions of the other species' pheromone, were compared with those with virgin females of the target species or unbaited controls. These experiments were carried out with jerry-can traps.

Results showed that addition of varying amounts of dodecyl (*E*)-2-butenoate to 0.1 mg decyl (*E*)-2-butenoate in vials did not significantly affect its attractiveness to *C. puncticollis* males (Table 5). Almost no *C. brunneus* males were trapped with any combination of the two pheromone components.

In the reciprocal experiments involving adding varying amounts of decyl (*E*)-2-butenoate to 0.1 mg dodecyl (*E*)-2-butenoate in septa, as little as 0.001 mg of the former, i.e., 1% of total pheromone, significantly reduced catches of

TABLE 4. CATCHES OF SWEETPOTATO WEEVILS IN 5-LITER JERRY-CAN TRAPS BAITED WITH VIRGIN FEMALE *C. brunneus* WEEVILS OR DODECYL (*E*)-2-BUTENOATE DISPENSED FROM POLYTHENE VIALS OR RUBBER SEPTA (5 REPLICATES; 5 NIGHTS)

Experiment/lure	Mean catch/trap/night (±SE)[a]	
	C. puncticollis	*C. brunneus*
March 1995		
Control (tuber piece)	2.3 ± 0.8 b	0.1 ± 0.1 c
10 virgin females + tuber piece	1.4 ± 0.6 b	21.5 ± 6.7 a
0.01-mg septum	8.1 ± 2.3 a	0.6 ± 0.3 bc
0.1-mg septum	13.6 ± 4.4 a	1.8 ± 0.6 b
1.0-mg septum	6.0 ± 1.1 a	2.0 ± 0.6 b
Slope, *P* for dose–response[b]	−0.01c, 0.87	0.11b, 0.03
March 1995		
Control (tuber piece)	3.2 ± 1.2 c	0.8 ± 0.4 c
10 virgin females + tuber piece	2.0 ± 0.7 c	29.3 ± 7.9 a
0.01-mg vial	6.6 ± 1.7 b	0.2 ± 0.1 c
0.1-mg vial	9.6 ± 1.8 b	4.5 ± 1.4 b
1.0-mg vial	16.0 ± 3.7 a	15.4 ± 2.8 a
Slope, *P* for dose–response[b]	0.20b, 0.003	0.49a, <0.001
May 1996		
Control (tuber piece)	0.7 ± 0.4 d	0.6 ± 0.3 c
10 virgin females + tuber piece	1.1 ± 1.0 d	23.7 ± 8.6 a
0.01-mg septum	4.8 ± 1.6 c	0.8 ± 0.3 c
0.1-mg septum	7.5 ± 1.5 b	6.3 ± 2.0 b
1.0-mg septum	28.7 ± 6.3 a	7.6 ± 2.7 b
Slope, *P* for dose–response[b]	0.37b, <0.001	0.22b, 0.002
June 1997		
5 virgin females + tuber piece	6.3 ± 1.0 c	46.4 ± 6.3 b
10 virgin females + tuber piece	1.6 ± 0.2 d	78.0 ± 6.0 a
0.01-mg septum	9.0 ± 1.0 c	15.3 ± 1.7 c
0.1-mg septum	42.4 ± 3.0 b	61.0 ± 7.2 ab
1.0-mg septum	102.1 ± 6.8 a	40.2 ± 4.8 b
Slope, *P* for dose–response[b]	0.59a, <0.001	0.19b, 0.01

[a]Means within a column group followed by a common letter were not significantly different ($P < 0.05$ for LSD following ANOVA of log-transformed catches).

[b]Value of the regression coefficient b, and the significance of its departure from zero, found from the linear regression of $\log_{10}$-transformed catch on $\log_{10}$(dose) ($y = a + bx$) for three loadings of synthetic pheromone; coefficients within a column followed by a common letter indicate they are not significantly different [$P < 0.05$, by method of Bailey (1981, p. 97)].

C. brunneus males, although 0.0001 mg (0.1%) did not (Table 6). Amounts of decyl (*E*)-2-butenoate equivalent to 10% of total pheromone or more completely suppressed catches of *C. brunneus* males, while increasing amounts of this compound generally produced higher catches of *C. puncticollis* males.

Time-of-Capture Experiments. The majority of *C. puncticollis* and *C. brun-*

TABLE 5. CATCHES OF SWEETPOTATO WEEVILS IN 5-LITER JERRY-CAN TRAPS BAITED WITH DECYL (*E*)-2-BUTENOATE AND DODECYL (*E*)-2-BUTENOATE IN POLYTHENE VIALS OR VIRGIN FEMALE *C. puncticollis* WEEVILS (4 REPLICATES; 5 NIGHTS)

Decyl (*E*)-2-butenoate (mg)	Dodecyl (*E*)-2-butenoate (mg)	Mean catch/trap/night (±SE)[a]	
		C. puncticollis	*C. brunneus*
September 1995			
0.1		13.1 ± 3.5 a	0.1 ± 0.1
0.1	0.01	15.4 ± 6.0 a	0
0.1	0.1	18.2 ± 5.5 a	0
10 virgin female *C. puncticollis*		4.0 ± 1.2 b	0.1 ± 0.1
control (tuber piece)		0.4 ± 0.2 c	0
February 1997			
0.1		31.5 ± 4.2 a	0
0.1	0.0001	37.3 ± 6.2 a	0
0.1	0.001	39.2 ± 6.6 a	0
0.1	0.01	42.8 ± 6.9 a	0
Control (blank)		0.4 ± 0.2	0.1 ± 0.1

[a]For *C. puncticollis*, means within a column group followed by a common letter were not significantly different ($P < 0.05$ for LSD following ANOVA of log-transformed catches). Controls omitted from ANOVA of February 1997 data.

neus males were trapped overnight between 17:00 and 08:00 hr (Figure 1, top). The proportion trapped during daylight hours was negligible (3%) in the case of *C. puncticollis*, but with *C. brunneus* it averaged 21%, and there was relatively little day-to-day variation in these trends. More detailed observations (Figure 1, bottom) showed that the great majority of *C. puncticollis* (>90%) were trapped between midnight and 04:00 hr but most *C. brunneus* (also > 90%) were trapped between 16:00 and 21:00 hr. Thus, there was a clear separation in the times of peak catches of the respective species, although small numbers of both (<5%) were captured between 21:00 hr and midnight.

These timings were refined further. The largest numbers of *C. puncticollis* males (80% of the overnight total) were trapped in the two 1-hr periods between 01:00 and 03:00 hr (Figure 2, top). When catches of *C. brunneus* males were recorded at 2-hr intervals between 09:00 and 21:00 hr, the proportion captured during daylight hours up to 17:00 hr was very similar to the corresponding value in Figure 1 (top), and these individuals were captured at any time after 09:00 hr with roughly equal probability (Figure 2, middle). Most *C. brunneus* males were captured between 17:00 and 21:00 hr, but the exact timing of peak catches varied. During separate observations of captures recorded at hourly intervals between 16:00 and 21:00 hr, the general trend was for the majority to occur from 19:00 to 20:00 hr (Figure 2, bottom). However, on one of the six evenings of observation, 59% of males were trapped between 16:00 and 17:00 hr, and similar results were

TABLE 6. CATCHES OF SWEETPOTATO WEEVILS IN 5-LITER JERRY CAN TRAPS BAITED WITH DODECYL (*E*)-2-BUTENOATE AND DECYL (*E*)-2-BUTENOATE IN RUBBER SEPTA OR VIRGIN FEMALE *C. brunneus* WEEVILS (4 REPLICATES; 5 NIGHTS)

Dodecyl (*E*)-2-butenoate (mg)	Decyl (*E*)-2-butenoate (mg)	[a]Mean catch trap^{-1} night^{-1} (±SE)		ratio
		C. puncticollis	*C. brunneus*	*C.b./C.p.*
September 1995				
0.1		12.4 ± 5.0 b	14.7 ± 3.0 a	1.2
0.1	0.01	6.5 ± 1.4 b	0.1 ± 0.1	<0.1
0.1	0.1	29.8 ± 6.4 a	0.1 ± 0.1	<0.1
10 virgin female *C. brunneus*		0.1 ± 0.1	13.6 ± 3.5 a	94.0
Control (tuber piece)		0	0.4 ± 0.1 b	
February 1997				
0.1		13.1 ± 2.7 bc	7.8 ± 2.3 a	0.6
0.1	0.0001	18.9 ± 4.7 b	9.3 ± 1.6 a	0.5
0.1	0.001	20.6 ± 3.5 b	4.5 ± 0.8 b	0.2
0.1	0.01	50.9 ± 5.8 a	0.5 ± 0.2 c	<0.1
Control (blank)		0.3 ± 0.1	0.1 ± 0.1	

[a]Means within a column group followed by a common letter were not significantly different ($P < 0.05$ for LSD following ANOVA of log-transformed catches). Where no letter appears after a mean, that treatment was omitted from the appropriate ANOVA.

observed on two evenings when the total catches were too low for inclusion with the present results.

Measurement of Release Rates of Synthetic Pheromones from Dispensers. Amounts of the synthetic pheromone components, decyl (*E*)-2-butenoate and dodecyl (*E*)-2-butenoate, remaining at intervals in polyethylene vials and rubber septa exposed under constant laboratory conditions at 27°C and 8 km/hr windspeed are shown in Figure 3. Assuming there was no pheromone degradation in the dispensers under these conditions, the rate of disappearance is equivalent to release rate, and, as expected, release of the higher molecular weight, less volatile dodecyl (*E*)-2-butenoate was slower than that of decyl (*E*)-2-butenoate. Release rates were first order in each case, and, for the former, release was slower from the septa (half-life 115.5 days) than from the vials (half-life 49.2 days). For the latter, release rates for the two types of dispenser were more similar, but release was slower from the vials (half-life 19.3 days) than from the septa (half-life 14.6 days). Most field experiments were carried out over five-day periods with fresh lures. Release rates over the first five days under laboratory conditions were relatively constant (Figure 3) and for an initial loading of 1 mg can be calculated from the above data as 0.25 μg/hr and 0.64 μg/hr for the dodecyl (*E*)-2-butenoate in septa and vials, respectively, and 1.91 μg/hr and 1.64 μg/hr are the corresponding rates for decyl (*E*)-2-butenoate.

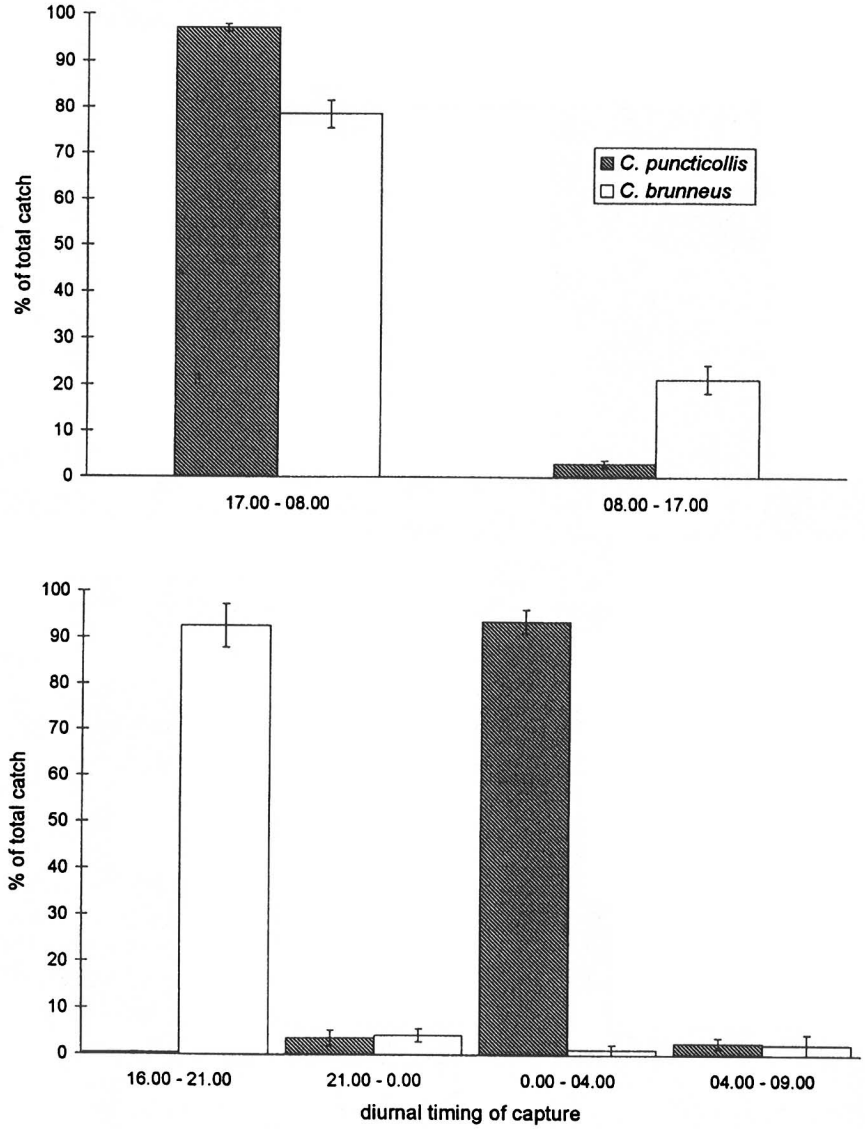

FIG. 1. Diurnal timing of capture of *Cylas brunneus* and *C. puncticollis* males in traps baited with their respective synthetic sex pheromones; top—data from observation sets 1 and 6 (four nights only) respectively; bottom—data from sets 2 and 7–9, respectively (vertical bars indicate standard errors).

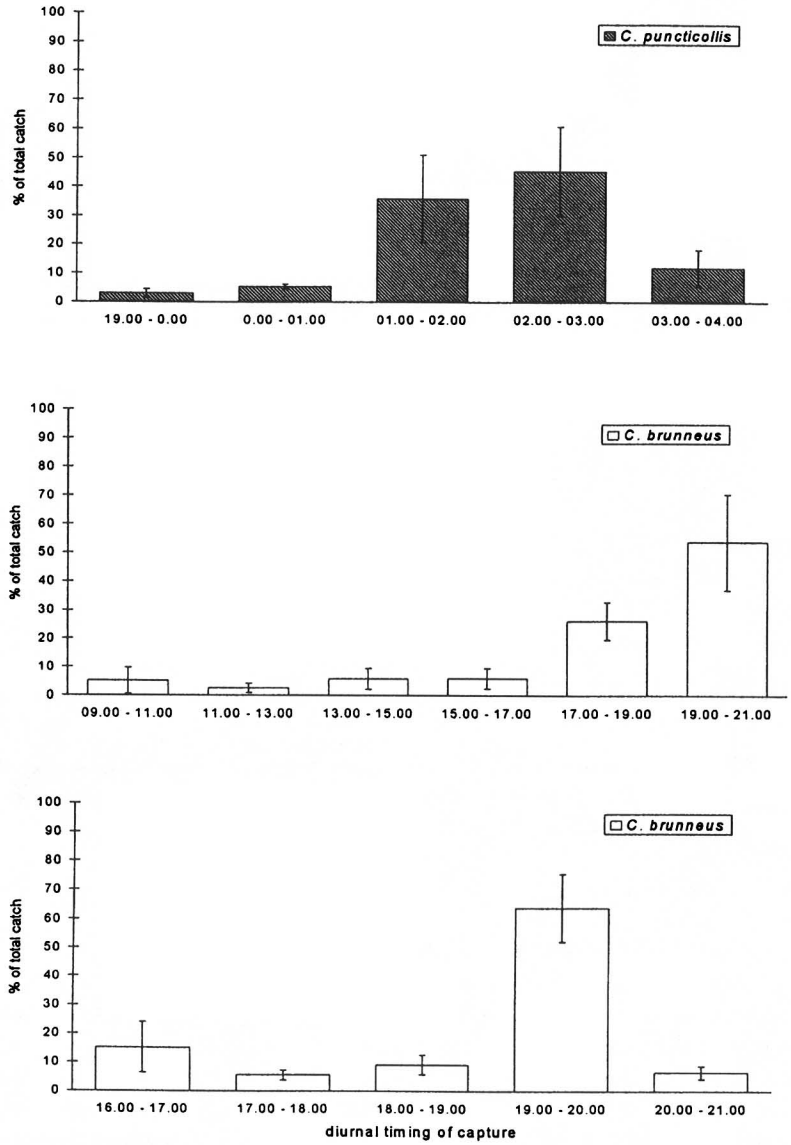

FIG. 2. Diurnal timing of capture of *Cylas brunneus* and *C. puncticollis* males in traps baited with their respective synthetic sex pheromones; top—data for *C. puncticollis* from observation sets 8 and 9; middle—data for *Cylas brunneus* from observation sets 4 and 5; bottom—data for *Cylas brunneus* from observation sets 3–5 (vertical bars indicate standard errors).

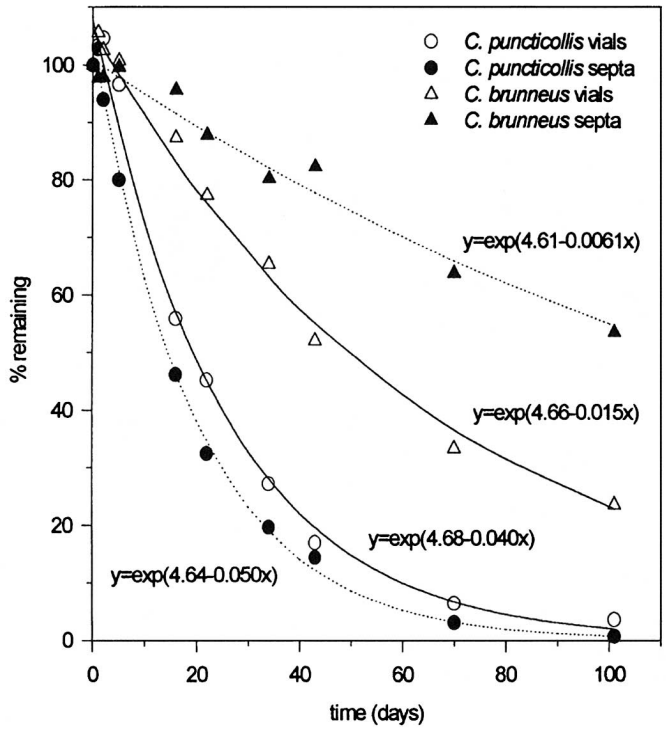

FIG. 3. Percentage of decyl (*E*)-2-butenoate (*C. puncticollis* pheromone) and dodecyl (*E*)-2-butenoate (*C. brunneus*) remaining in polythene vials and rubber septa in the laboratory wind tunnel at 27°C and 8 km/hr windspeed.

Trapping of C. formicarius *in Indonesia.* In the first stage of the experiment when no traps baited with *C. formicarius* lure were deployed, catches of *C. formicarius* males in unbaited traps or traps baited with decyl (*E*)-2-butenoate were negligible, but significant catches were obtained in traps baited with dodecyl (*E*)-2-butenoate (Table 7). However, in the second stage, although catches with dodecyl (*E*)-2-butenoate lures were still significantly higher than those in traps baited with decyl (*E*)-2-butenoate, they were less than 0.4% of the catches in traps baited with lures containing the *C. formicarius* pheromone, (*Z*-3-dodecenyl (*E*)-2-butenoate. There was some evidence that catches were generally increased by the presence of the *C. formicarius* traps, presumably as large numbers of males were drawn into the experimental area. Thus catches of 48 and 121 males were observed in unbaited traps when these were next to traps baited with *C. formicarius* lures.

TABLE 7. CATCHES OF *C. formicarius* IN WATER-PAN TRAPS BAITED WITH PHEROMONES OF *C. puncticollis*, *C. brunneus*, AND *C. formicarius* DISPENSED FROM RUBBER SEPTA IN INDONESIA, OCTOBER 1997[a]

Experimental period	Mean catch/trap/night of *C. formicarius* (±SE)[b]			
	(*E*)-2-Butenoate			Control
	Decyl	Dodecyl	(*Z*)-3-Dodecenyl	(blank)
Days 1–3	0.3 ± 0.3 b	10.4 ± 2.8 a	—	0
Days 4–7	2.4 ± 1.0 c	6.4 ± 1.7 b	1820.1 ± 381.1 a	11.1 ± 7.9 b

[a]Three replicates, three nights for days 1–3; four replicates, four nights for days 4–7.
[b]Means within a row followed by a common letter were not significantly different ($P < 0.05$ for LSD following ANOVA of log-transformed catches).

DISCUSSION

Hall et al. (unpublished data) showed that virgin female *C. puncticollis* weevils produced decyl (*E*)-2-butenoate and female *C. brunneus* weevils produced the dodecyl analog. Both compounds elicited electroantennographic (EAG) responses from males of both species. The results here show that decyl (*E*)-2-butenoate attracts male *C. puncticollis* weevils and that dodecyl (*E*)-2-butenoate attracts male *C. brunneus* weevils. However, whereas the former compound is highly specific for *C. puncticollis*, the latter attracts both *C. brunneus* and *C. puncticollis* males. Furthermore, addition of even small amounts of decyl (*E*)-2-butenoate to the dodecyl (*E*)-2-butenoate completely eliminates its attractiveness to *C. brunneus* males. Thus, although both compounds stimulate receptors on the antennae of both species, their behavioral effects are very different.

The degree of cross-attraction of *C. puncticollis* males by the pheromone component of *C. brunneus*, dodecyl (*E*)-2-butenoate, varied with the type of pheromone dispenser used. Rubber septa gave higher selectivity for *C. brunneus* males than polyethylene vials, and release rate data showed that release of dodecyl (*E*)-2-butenoate was slower from the septa than from the vials for a given initial loading. The higher selectivity observed is consistent with Roelofs' (1978) threshold hypothesis, by which higher release rates are required to attract species with the "wrong" pheromone blend. However, this explanation is not totally convincing, as selectivity of attraction of *C. brunneus* was not consistently increased at lower initial loadings and hence lower release rates of pheromone from the septa [cf. catches of the two species with dodecyl (*E*)-2-butenoate across the range of doses (Table 4)]. Attraction of *C. puncticollis* males by decyl or dodecyl (*E*)-2-butenoate was generally greater when the pheromone was dispensed from polyethylene vials rather than septa.

Although dodecyl (*E*)-2-butenoate, the pheromone component produced by

female *C. brunneus* weevils, attracts males of both species, species specificity of mating is aided by a clear temporal separation of male response to the pheromones. *C. puncticollis* males respond to pheromone between 01:00 and 03:00 hr, while *C. brunneus* males respond mainly between 19:00 and 21:00 hr, but with some responsiveness shown throughout daylight hours. Smit et al. (1994) noted that *C. puncticollis* only mated at night, whereas *C. brunneus* mated only during daylight, and this suggests that the diel timing of male sexual responsiveness is probably reinforced by a complementary periodicity in production of pheromone by the females. Thus traps baited with virgin female weevils trapped almost exclusively conspecific males, and species-specific mating is ensured in nature.

Jansson et al. (1990, 1992) found a consistent positive dose–response for *C. formicarius* for a wide range of doses (1×10^{-6}–10 mg) of its synthetic pheromone in rubber septa lures. Regression coefficients generally varied between 0.18 and 0.44 (1.5- to 2.75-fold increase in catches for each 10-fold increase in dose). We found dose–response relationships for *C. puncticollis* and *C. brunneus* were more variable, in both form and slope, over a smaller range of doses. Low or variable weevil populations may partly account for this in some instances. There was evidence that the dose–response was related to dispenser type. For *C. puncticollis*, the two values obtained when using decyl (*E*)-2-butenoate in vials were much higher than the almost nonexistent dose–response observed when the same compound was dispensed from septa. For *C. brunneus* with dodecyl (*E*)-2-butenoate the regression coefficient of 0.49 found with polythene vial dispensers was significantly higher than the three values observed with septa dispensers, although in these latter cases it is acknowledged that the apparently sigmoid relationships are not adequately described by a straight line. Although there was no similar pattern for *C. puncticollis* responding to dodecyl (*E*)-2-butenoate, these results do suggest that release rate might be important, at least for *C. brunneus*, since release rates of dodecyl (*E*)-2-butenoate from the two types of dispenser differed markedly.

In these field studies, traps baited with decyl (*E*)-2-butenoate and with virgin female *C. puncticollis* caught similar numbers of *C. puncticollis* males, while traps baited with dodecyl (*E*)-2-butenoate often caught fewer *C. brunneus* males than those baited with virgin female *C. brunneus* weevils. These findings contrast strongly with comparable results for *C. formicarius* in which traps baited with 0.01 mg synthetic lures caught 275 males per night while traps baited with three virgin females captured only five males per night (Jansson et al., 1991). There is no clear explanation for the disparity in the relative attractiveness of synthetic and natural pheromones among the three *Cylas* species, although an obvious possibility is that the synthetic pheromone of *C. brunneus*, in particular, is deficient in some way. Hall et al. (unpublished data) could find no evidence for any minor pheromone components in linked GC-EAG analyses of female *C. brunneus* volatiles, although their existence cannot be ruled out. In view of the findings concerning the timing of activity of males, the lack of specificity of attraction of the dodecyl (*E*)-2-butenoate

does not require postulation of additional components in the pheromone of *C. brunneus* to make attraction and mating species-specific. Attraction of both *C. puncticollis* and *C. brunneus* does seem to be very dependent upon pheromone release rate and the type of dispenser used for the synthetic pheromone, so that physical rather than chemical factors may explain differences in attractiveness, while other factors, behavioral or ecological, might also play a role.

Whereas *C. puncticollis* and *C. brunneus* are restricted to Africa, *C. formicarius* has a much more cosmopolitan distribution, although it has been reported in only a few African countries. Smit et al. (1994) and Sathula (1993) reported that (*Z*)-3-dodecenyl (*E*)-2-butenoate, the pheromone of *C. formicarius*, does not attract either of the African species. Our results here showed that although dodecyl (*E*)-2-butenoate, the pheromone component of *C. brunneus*, is significantly attractive to *C. formicarius* males, this attractiveness is less than 0.4% that of the *C. formicarius* pheromone. Dodecyl (*E*)-2-butenoate differs from the *C. formicarius* pheromone by absence of the (*Z*)-3-double bond whereas decyl (*E*)-2-butenoate, which was not significantly attractive to *C. formicarius* males, differs by absence of the double bond and a shorter chain length.

These results and those of Hall et al. (unpublished data) indicate that the pheromones of *C. puncticollis* and *C. brunneus* probably consist of the single components decyl (*E*)-2-butenoate and dodecyl (*E*)-2-butenoate, respectively. Polyethylene vials are recommended as dispensers for decyl (*E*)-2-butenoate in traps for *C. puncticollis*, while rubber septa loaded with dodecyl (*E*)-2-butenoate are more selective for *C. brunneus* males than polyethylene vials. Lures for both species cannot be combined in the same trap because of the strong inhibition of attraction of *C. brunneus* by decyl (*E*)-2-butenoate. The optimal loading for both species appears to be 0.1 mg, and the lures remain attractive for at least eight weeks in the field (Smit et al., 1997). These findings will have implications for monitoring and control programs for these pests based on pheromones (Laboke et al., 1997).

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THE FATE AND EFFECTS OF INGESTED HYDROLYZABLE TANNINS IN *Porcellio scaber*

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Abstract—When adults of *Porcellio scaber* fed on litter prior to an artificial diet containing 5% of commercially available tannic acid, 55% of the ingested galloylglucose esters was excreted unchanged, about 25% was hydrolyzed, and 20% was oxidized during the gut passage. After reducing the counts of microorganisms in the gut of *P. scaber*, the data obtained indicated an important role of ingested platable microorganisms in hydrolyzing gallotannins. Oxidation of phenolics appeared to be mainly due to the endosymbiotic bacteria of the hepatopancreas. Microbial counts in the hindgut were strongly reduced by ingested galloylglucose esters, while gallic acid in the diet (2%) reduced the number of palatable fungi and bacteria less strikingly, and increased the total number of the gut microbiota. Hepatopancreatic bacteria were only slightly affected by ingested tannic acid, since the hepatopancreas contained only few galloylglucose esters. This may be due to the permeability of the hindgut cuticle: the cuticle of the anterior hindgut was freely permeable to gallic acid, while it was nearly impermeable to larger polyphenols. The cuticle of the posterior hindgut was permeable to only about 4% of the gallic acid present in the hindgut lumen. The results are discussed with respect to potentially harmful effects of ingested hydrolyzable tannins and their digestion in *Porcellio scaber*.

Key Words—Terrestrial isopods, Oniscidea, *Porcellio scaber*, phenolics, hydrolysis, oxidation, detoxification, hindgut cuticle, gut microbiota, hepatopancreatic bacteria.

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INTRODUCTION

Tannins are naturally occurring water-soluble polyphenolic compounds capable of complexing proteins in vitro (Hagerman and Butler, 1991). The effects of hydrolyzable and condensed tannins on consumption and digestion in phytophagous insects have been examined in detail (summarized in Mole and Waterman, 1987; Bernays, 1981; Hagerman and Butler, 1991). Effective physiological counteractions have been described in the gut of caterpillars (e.g., Berenbaum, 1980; Martin et al., 1987). Hydrolysis of tannins may serve as a mechanism of detoxification (Bernays and Chamberlain, 1980; Hagerman and Butler, 1991). On the other hand, oxidative degradation of phenolics may lead to harmful quinones or reactive oxygen species (Appel, 1993).

In contrast to phytophagous insects, little is known about the influence of phenolic compounds in the litter on saprophagous soil animals. Although most phenolics are readily removed from the litter by leaching (Kuiters and Sarink, 1986), effects of condensed tannins—still present in the litter after leaching—on reproduction and mortality of terrestrial isopods (Isopoda: Oniscidea) have been demonstrated recently (Zimmer and Topp, 1997a). However, no data on hydrolytic detoxification or oxidative activation of hydrolyzable tannins in the gut of terrestrial isopods are available.

In phytophagous insects, the uptake of tannin components from the gut lumen may be prevented by the peritrophic envelope (Bernays and Chamberlain, 1980; Bernays et al., 1980; Barbehenn and Martin, 1992, 1994, 1995). The hindgut cuticle of terrestrial isopods (Lane, 1988) appears to have a similar composition as the peritrophic envelope in insects (Brandt et al., 1978; Stamm et al., 1978), and it apparently fulfills similar functions (cf. Fawcett, 1965).

The present study was conducted to determine the fate of ingested hydrolyzable tannins in the gut of the common terrestrial isopod, *Porcellio scaber* Latreille. Commercially available tannic acid served as a simple model compound that has previously been investigated in insects (e.g., Barbehenn and Martin, 1992, 1994).

METHODS AND MATERIALS

Gut of Terrestrial Isopods. The gut of *P. scaber* can be divided into three parts (Figure 1). Two pairs of midgut lobes (hepatopancreas) that secrete digestive fluids and absorb nutrients fill up the body cavity along the alimentary canal (Hassall and Jennings, 1975; Hames and Hopkin, 1989). The hepatopancreas is characterized by abundant endosymbiotic bacteria (Wood and Griffiths, 1988) that are involved in the digestion of cellulose (Zimmer and Topp, 1998a,b) and lignins (Zimmer and Topp, 1998c). In the hindgut, an anterior and a posterior

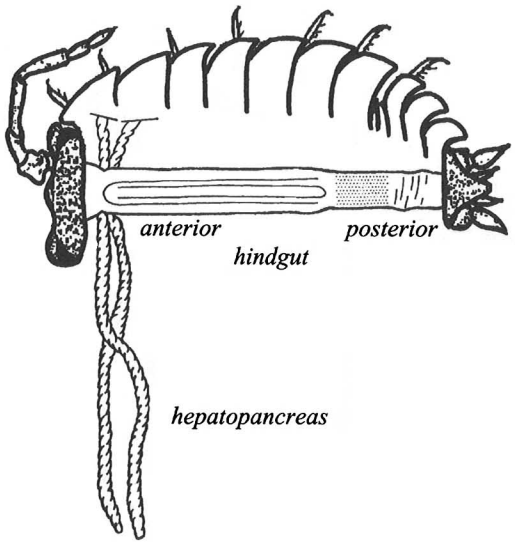

FIG. 1. Gut sections of terrestrial isopods (*Porcellio scaber*) referred to in the text.

part can be distinguished (e.g., Hassall and Jennings, 1975; Hames and Hopkin, 1989; cf. Figure 1). The anterior part is characterized by typhlosole infoldings and contains numerous microorganisms ingested with the food (Reyes and Tiedje, 1976; Zimmer and Topp, 1998b). The posterior part of the hindgut is a straight tube that can be subdivided morphologically, but was considered as one section in this study. It is in this hindgut section that bacterial proliferation takes place (Zimmer and Topp, 1998b).

Experimental Design. Individuals of *P. scaber* (Isopoda, Oniscidea) were collected in a floodplain forest near Cologne, Germany. In the laboratory, the woodlice were kept separately in small Petri dishes. Only adult specimens of about 20–30 mg fresh wt were used in experiments. All experiments were carried out at 15°C and a photophase of 16L : 8D.

Prior to the experiments, the isopods were fed with litter from the forest in which they were captured. By this method, the natural populations of gut microbiota were expected to be maintained. Microorganisms in the guts of terrestrial isopods have been described in detail (Reyes and Tiedje, 1976; Griffiths and Wood, 1985; Ullrich et al., 1991). Populations of gut microbiota are influenced when woodlice feed on sterile artificial diet or on diet containing antibiotics (Zimmer and Topp, 1998b). Thus, to alter the microbial counts and their activity in the gut of *P. scaber*, specimens were fed with a sterile chemically defined artificial diet modified after Carefoot (1984). This diet was rich in cellulose (30%), starch (25%), and simple carbohydrates (10% sucrose, and 5% each

of glucose, lactose, and maltose). Casein (15%) served as a nitrogen source. A mineral mix (5%) was added according to Retnakaran and Beck (1968).

The diet (1 g) was mixed thoroughly with agar (0.3 g) and suspended in hot distilled water (2 ml). After cooling, the diet (pH 5.4) was kept at 4°C for up to five days. To reduce microbial counts in the gut, antibiotics [1% w/w of a mixture of oxytetracycline (Terramycin), Tegostep, Dithane and sorbic acid] were used (Sigma, St. Louis, Missouri).

Gut Microbiota. After feeding on the described food sources, some specimens were examined to determine the influence of the food source on microbial counts in the gut. Counts of platable bacteria and fungi (platable microbial counts, PMC) were determined as described in Treves and Martin (1994). Since endosymbiotic bacteria of the hepatopancreas cannot be cultivated by this plating technique (Wood and Griffiths, 1988; Zimmer, 1998), platable microorganisms can be considered as ingested microbiota. Total microbial counts (TMC) that reveal additional information on nonplatable microbiota, including hepatopancreatic endosymbionts, were estimated by the acridine orange method (Francisco et al., 1973).

Phenolics in the Diet. For studies on the degradation of hydrolyzable tannins in the gut of *P. scaber*, the artificial diet was enriched with 5% of commercially available tannic acid (Sigma), resulting in a pH value of 5.5. A budget for gallic acid was determined with a diet containing 2% gallic acid (Sigma), pH 5.4.

During the feeding experiment, artificial diet (30 mg fresh wt; 10 mg dry wt) containing tannic acid was offered to individuals of *P. scaber* for five days. Feces from the previous food source (litter or artificial diet without phenolics) were removed from the Petri dishes twice a day. The feces derived from the experimental diet were collected quantitatively twice a day and were stored at −20°C until they were lyophilized and weighed. After five days of feeding, the remaining diet was removed, lyophilized, and weighed to estimate the amount of ingested diet (dry wt). From these values the amount of ingested gallotannins could be calculated by using a standard curve of fresh diet (dry wt). As a comparison between fresh diet and diet after five days of experimental treatment indicated, changes in phenolic content that were due to chemical breakdown and autoxidation were negligible. Litter was offered as subsequent food to collect further feces derived from the tannin containing diet that were easily distinguished by their color. Data of ingested diet and egested feces allowed the estimation of consumption rates (RCR) and digestibility (AD) according to Waldbauer (1968).

Specimens of *P. scaber* were dissected to extract the described sections of the gut. After lyophilizing the samples of diet, gut contents, and feces, the gallotannins were extracted three times in 1000 μl of 70% acetone containing 0.001 M ascorbic acid at 22 ± 1°C. Extracts were centrifuged (4 min; 13,600 *g*), and the supernatant solutions were pooled, concentrated under a stream of nitrogen,

and lyophilized. Lyophilized extracts were resolubilized in 500 μl of 23% v/v acetronitrile in double-distilled water containing 1% acetic acid. After filtration (0.45 μm, Gelman GHP), the qualitative and quantitative determination of the compounds of tannic acid was performed by reverse-phase HPLC with a Waters 10-μm C-18 column (4.6 × 250 mm). The mobile phase was 18% v/v acetonitrile in double-distilled water containing 1% acetic acid. The flow rate of the mobile phase was 1 ml/min. Samples (25-μl aliquots) were injected with an autoinjector (Shimadzu). Peaks were detected with a Shimadzu UV–visible detector (280 nm; 0.002 AUFS) and quantified with a Shimadzu C-R4A chromatopac. After correcting peak areas for interfering substances present in control samples, the amount of phenolics was calculated from the peak area by using standard curves of tannic acid and gallic acid. In this study, the fate of four tannic acid compounds that eluted in the range of 3–10 min was noted (Figure 2a). One of these compounds (peak A, Figure 2a) was identified as gallic acid by standards. According to Barbehenn and Martin (1994), gallic acid is present in commercially available tannic acid as an impurity. The other compounds of tannic acid are considered as galloylglucose esters (cf. Barbehenn and Martin, 1992), and are named peaks B, C, and D (Figure 2a). According to Barbehenn et al. (1996), the number of galloyl groups, and thus, the molecular weight of these compounds is positively correlated with their retention time.

To determine the extent of hydrolysis of gallotannins in the gut of *P. scaber*, the ingested and egested amounts of gallic acid and galloylglucose esters were used for a calculation of the tannin budget as described in Barbehenn et al. (1996). The amount of oxidized gallotannins was estimated from the difference between the total tannic acid ingested and the sum of the percentage of gallotannins recovered and hydrolyzed. Furthermore, by measuring the amounts of soluble brown pigments in the feces of isopods fed on tannic acid (Barbehenn et al., 1996) relative data on the extent of tannin oxidation was obtained.

Permeability of Gut Cuticle. Isopods ($N = 20$) were fed with diet containing tannic acid as described above. An additional 10 adults fed on artificial diet without phenolics served as a control. Either the anterior hindgut and the mid-hindgut, or the mid-hindgut and the posterior hindgut were taken for the subsequent measurements. Under a dissecting microscope, these parts of the hindgut, containing the ingested artificial diet, were ligated with fine silk sutures (Barbehenn and Martin, 1995). Afterwards, a small hole (0.1 ± 0.02 mm^2) was cut into the tissue of either the posterior hindgut or the ventral anterior hindgut, where there are no typhlosole canals, to expose the cuticle. To measure the permeation of compounds in the gut lumen, the prepared guts were transferred into 1 ml of an incubation solution (3 mM NaCl, 227 mM KCl, 300 mM fructose, 175 mM NaH_2PO_4, 25 mM Na_2HPO_4) (Giordana and Sacchi, 1978) that was roughly consistent with hemolymph composition (Wright et al., 1997; Ziegler and Scholz, 1997) and stirred for 2 hr at 22 ± 1°C. As controls, indicating that

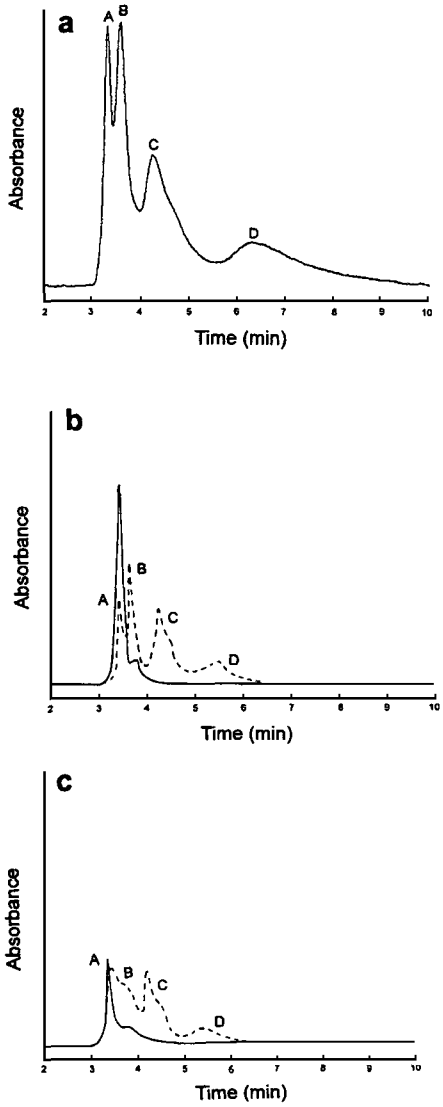

FIG. 2. Chromatogram of a tannic acid standard (a), illustrating the compounds observed by reverse-phase HPLC, and representative chromatograms of (b): the anterior hindgut (dotted line) and the surrounding incubation solution (solid line), and (c): the posterior hindgut (dotted line) and the surrounding incubation solution (solid line), after substracting control chromatograms of specimens fed on diet without tannic acid. Peak A: gallic acid; peaks B–D: galloylglucose esters. No compounds eluted before 2 min.

permeation of phenolics through the hindgut cuticle was observed, but not leaching of phenolics at the sutured ends, five gut samples without exposed cuticles were placed in the incubation solution. The incubation solutions, containing the permeated compounds of tannic acid, were frozen at −20°C and subsequently lyophilized. Phenolics were extracted from the gut contents as described above.

The permeability of the hindgut cuticle of *P. scaber* to gallic acid and different galloylglucose esters was determined as the percent of these TA compounds in the incubating solution, compared to the total amount initially present in the anterior or posterior hindgut (Figure 2b and c) after feeding on artificial diet containing tannic acid: permeability = [content in solution/(content in gut + content in solution)].

Statistics. Most of the data showed deviations from the model of normal distribution. To treat all data in the same manner, generally nonparametric statistics were used. Hence, results are presented as median (M) ± median absolute deviation (MAD). Comparison of different treatments concerning consumption and digestion of food and the formation of brown pigments were conducted by the multivariate Kruskal-Wallis H test. Pairwise differences between treatments were localized with the Mann-Whitney U test. In the case of results given in percentages, data were transformed (x' = arc sin $\sqrt{x}$) and subsequently compared as described above.

RESULTS

Effects of Tannic Acid on Consumption and Digestion. Consumption and assimilation of the offered food are given in Table 1. After foraging on litter, relative consumption rates (RCR) were reduced by feeding on artificial diet with tannic acid (TA) compared to diet without TA ($P < 0.001$). A further reduction of RCR occurred when *P. scaber* had previously fed on artificial diet ($P < 0.001$). Antibiotics did not show any effect on the consumption rate. The effects of ingested gallic acid (GA) on RCR differed only slightly from those of TA, when the isopods had fed on litter ($P < 0.1$), and no differences between the effects of GA and TA on RCR was observed in isopods fed on artificial diet (Table 1).

With respect to the approximate digestibility (AD) of the diet, ingested tannic acid did not influence *P. scaber* when the isopods had previously fed on litter. However, foraging on artificial diet significantly reduced AD when TA was ingested ($P < 0.05$). Antibiotics in the diet did not show any effects on AD.

GA in the diet strikingly increased AD compared to the corresponding TA samples ($P < 0.001$) and in comparison to isopods feeding on artificial diet without phenolics ($P < 0.01$). Again, antibiotics did not alter AD.

Effects of Tannic Acid on Gut Microbiota. Isopods that fed on artificial diet had reduced numbers of gut microbiota compared to those that fed on litter, while

TABLE 1. CONSUMPTION (RCR) AND DIGESTIBILITY (AD) OF DIFFERENT FOOD SOURCES BY YOUNG ADULT (20–30 mg) *Porcellio scaber*[a]

Preexperimental diet	Content of phenolics in diet[b]	RCR	AD
Leaf litter	0 %	0.20 ± 0.10a	30 ± 20a
Leaf litter	5 % TA	0.09 ± 0.05b	23 ± 14a
Artificial diet[b]	5 % TA	0.03 ± 0.02c	17 ± 7b
Diet[b] + antibiotics	5 % TA	0.03 ± 0.02c	10 ± 9b
Leaf litter	2 % GA	0.04 ± 0.02d	65 ± 5c
Artificial diet[b]	2 % GA	0.02 ± 0.01c	86 ± 9d
Diet[b] + antibiotics	2 % GA	0.02 ± 0.01c	78 ± 13d

[a]TA: commercially available tannic acid; GA: gallic acid. Data are presented as median ± MAD ($N = 9$). The results of statistical comparison ($\alpha = 0.05$) are indicated by letters.
[b]Artificial diet after Zimmer and Topp (1998b).

the number of microorganisms in the hepatopancreas was not changed (Table 2). Antibiotics in the diet resulted in further decrease of microbial counts in the hindgut ($P < 0.001$), and in addition, reduced the number of hepatopancreatic bacteria ($P < 0.05$).

By contrast, gallic acid in the diet increased the number of these bacteria ($P < 0.01$), while tannic acid slightly reduced microbial counts of the hepatopancreas ($P < 0.05$). In the hindgut, tannic acid reduced the number of platable microbiota ($P < 0.001$). In the anterior hindgut, total microbial counts were

TABLE 2. INFLUENCE OF ARTIFICAL DIET (ad/) ARTIFICAL DIET WITH 1% ANTIBIOTICS (ad/+), 5% TANNIC ACID (ad/TA) OR 2% GALLIC ACID (ad/GA) ON NUMBERS OF MICROBIOTA IN MIDGUT LOBES (Hepatopancreas) AND ANTERIOR AND POSTERIOR HINDGUT OF *Porcellio scaber*[a]

	Total microbial counts			Platable microbial counts		
Food	Hepatopancreas	Anterior hindgut	Posterior hindgut	Hepatopancreas	Anterior hindgut	Posterior hindgut
ad/[b]	99^a	71^b	109^a	–	13^b	73^b
ad/+[c]	84^b	32^c	47^b	–	3^c	12^c
ad/TA[c]	72^b	151^d	57^b	–	6^c	20^c
ad/GA[c]	145^c	306^e	173^c	–	38^b	76^b

[a]Data of microbial counts after feeding the above-mentioned diets are given as the percent of the mean microbial counts of isopods fed on leaf litter ($N = 15$). The results of statistical comparison of absolute numbers of microbiota ($\alpha = 0.05$) are indicated by letters (microbial counts of isopods fed on litter are indicated as a).
[b]As a proportion of litter-feeding isopods.
[c]As a proportion of ad/-feeding isopods.

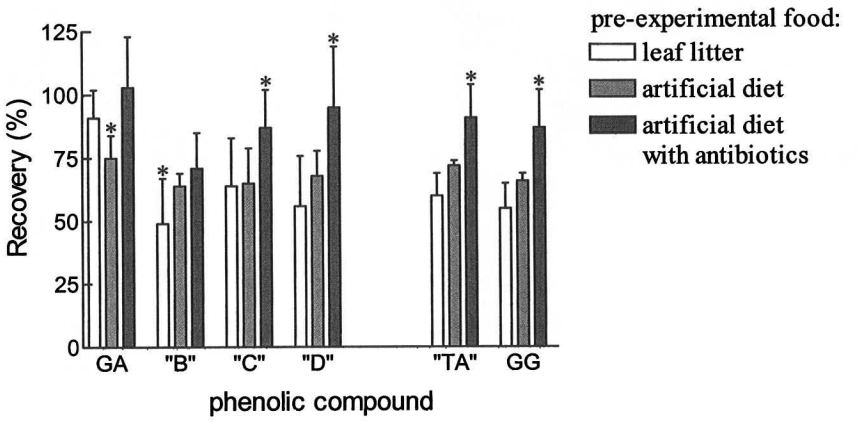

FIG. 3. Recovery of compounds of tannic acid (TA) from the feces of *Porcellio scaber* having fed on TA-containing artificial diet after different pre-experimental diets. GA: gallic acid; B–D: galloylglucose esters (cf. Figure 2a); TA: commercially available tannic acid, including GA; GG: total of galloylglucose esters B–D. Asterisks mark differences between one sample and the two of the comparable samples ($\alpha = 0.05$; $N = 10$).

increased by feeding on tannic acid and gallic acid ($P < 0.01$). The latter compound led to increased TMC in the posterior hindgut, too ($P < 0.001$), while TMC significantly decreased after feeding on tannic acid ($P < 0.001$). Platable bacteria and fungi in the hindgut were affected less strikingly by gallic acid than by tannic acid (Table 2).

Degradation of Tannic Acid during Gut Passage. When *P. scaber* had fed on litter prior to the experiments, about $60 \pm 9\%$ of the ingested tannic acid could be recovered from the feces. However, the observed TA compounds showed remarkable differences concerning the recovery from the feces (Figure 3). About 25% was hydrolyzed in the gut of *P. scaber*.

The distribution of TA compounds in the gut of *P. scaber* is shown in Table 3. The hepatopancreas contained about 12% of the ingested GA, but only small amounts of peak B (Figure 2a). No other compounds of tannic acid could be detected in the hepatopancreas. The anterior and posterior hindguts did not differ in their GA content, but the latter gut section did not contain any peak B, in contrast to the anterior hindgut. The contents of the other galloylglucose esters (peaks C and D in Figure 2a) were similar in both hindgut sections. Foraging on artificial diet reduced the extent of hydrolysis of TA to about 16% of the ingested amount (Figure 3).

The gallic acid content of the hepatopancreas (Figure 4) increased with decreasing counts of ingested (= platable) microorganisms in the hindgut [isopods fed on artificial diet (Table 2; $P < 0.05$)]. A similar effect of pre-ex-

TABLE 3. PERMEABILITY OF VARIOUS REGIONS OF HINDGUT CUTICLE TO COMPOUNDS OF TANNIC ACID[a]

Hindgut	GA (%)	Peak B (%)	Peak C (%)	Peak D (%)
Anterior	85 ± 10a	20 ± 5c	0e	0e
Posterior	4 ± 3b	2 ± 2d	0e	0e

[a]GA: gallic acid; peaks B, C, and D: galloylglucose esters. Data are given as median ± MAD ($N = 9$) of the percent recovered from an incubation solution to the amount of the compounds initially present in the corresponding section of the hindgut. The results of statistical comparison ($\alpha = 0.05$) are indicated by letters.

perimental artificial diet was observed concerning peak B ($P < 0.01$). None of the other galloylglucose esters of tannic acid were detected in the hepatopancreas of *P. scaber* after feeding on artificial diet prior to the experiment. The amount of recovered galloylglucose esters in the hindgut increased with decreasing microbial counts in the gut ($P < 0.05$). However, no peak B was detected in the posterior hindgut. When the isopods fed on diet containing antibiotics prior to the experiments, all of the ingested GA (103 ± 20%) was excreted via the feces (Figure 3). Only 6% of ingested TA was hydrolyzed in this case.

The content of gallic acid and peak B in the hepatopancreas (Figure 4) strongly increased with decreasing counts of bacteria in the hepatopancreas [isopods fed on antibiotics (Table 2; $P < 0.01$)]. Galloylglucose esters other than peak B did not enter the hepatopancreas. Feeding on antibiotics prior to the experiment resulted in a significantly increased content of GA in the anterior hindgut ($P < 0.01$), but caused weak changes in the contents of galloylglucose esters in this hindgut section: only the content of peak B significantly decreased ($P < 0.01$). In contrast to pre-experimental food without antibiotics, about one fourth of the ingested peak B was found in the posterior hindgut. Additionally, a higher proportion of other galloylglucose esters was recovered ($P < 0.05$).

The oxidation of phenolics during the gut passage was indicated by brown pigments present in the feces of isopods feeding on tannic acid. While there were no brown pigments in control diet without phenolics and in feces from isopods fed on this diet, small amounts of brown pigments were detected in the diet containing TA (Figure 5). Significantly higher absorbance was measured in the extract of feces obtained from isopods that had fed on litter ($P < 0.01$) and those that had fed on artificial diet prior to the experiment ($P < 0.01$). After feeding on antibiotics, the feces contained significantly less brown pigments ($P < 0.01$), but still had higher absorbance than was obtained from the diet alone ($P < 0.05$).

Permeability of Hindgut Cuticle. The anterior and posterior hindgut differed remarkably in terms of the permeability of their cuticle (Table 3). While nearly all (75–95%) of the gallic acid initially present in the anterior hindgut

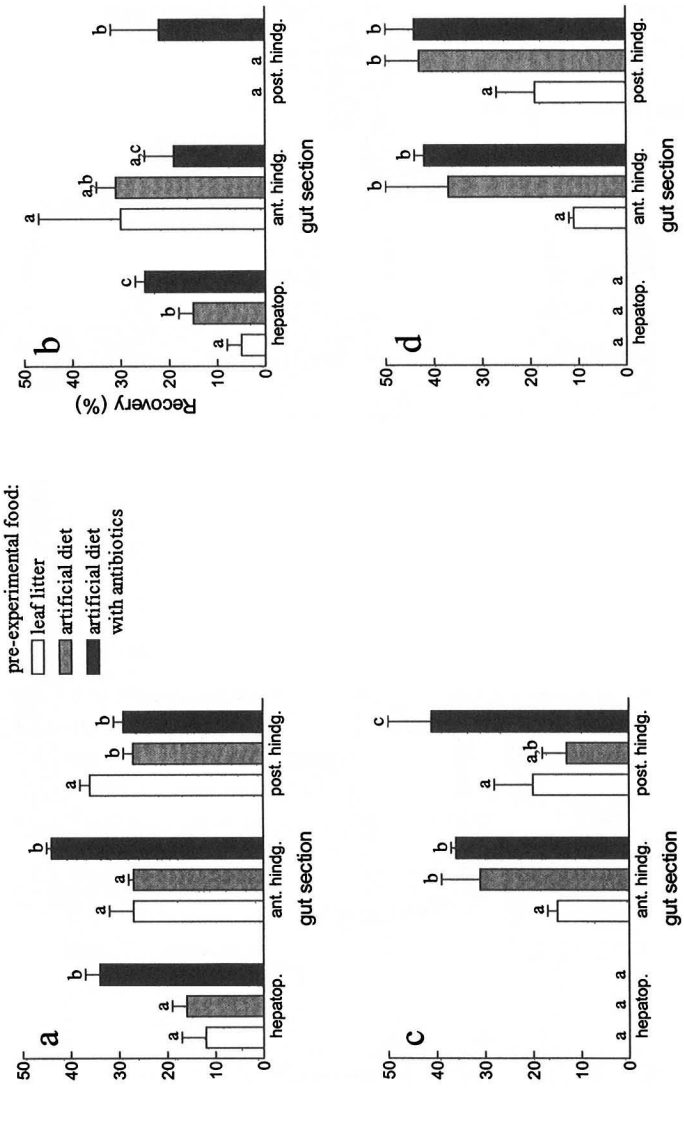

FIG. 4. Recovery of tannic acid compounds (cf. Figure 2a) (a): gallic acid; (b): peak B; (c): peak C; (d): peak D from the gut of *Porcellio scaber* (hepatop.: midgut lobes; ant. hindg.: anterior hindgut; post. hindg.: posterior hindgut) fed on artificial diet containing 5% tannic acid (TA). Data are presented as median ± MAD of the percent of recovery of the ingested amount ($N = 9$). The results of statistical comparison (comparing samples of the same sections of the gut; $\alpha = 0.05$) are indicated by letters.

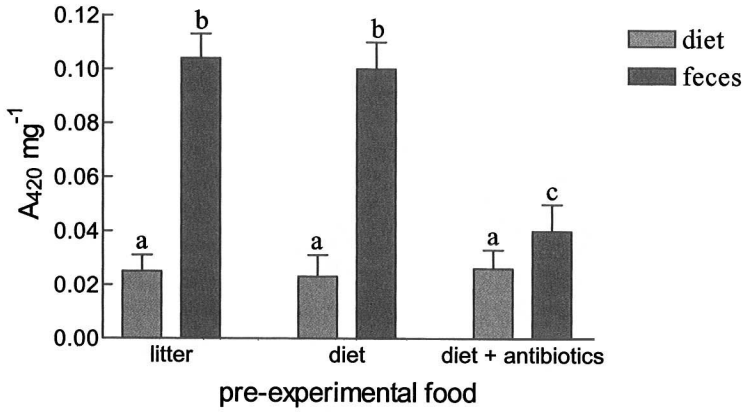

FIG. 5. Brown pigments, indicating phenol oxidation, in tannic acid-containing diet and the feces of *Porcellio scaber* having fed this food source after different preexperimental diets. Data are presented as mean ± MAD of relative absorbance (λ = 420 nm) per mg (N = 10). The results of statistical comparison (α = 0.05) are indicated by letters.

was found in the surrounding incubation solution, significantly less of peak B permeated the anterior hindgut cuticle of *P. scaber* than gallic acid, and higher molecular weight galloylglucose esters did not at all pass through the anterior hindgut cuticle.

Only 2–10% of the gallic acid present in the posterior hindgut permeated the cuticle of this hindgut section. Little of peak B (0–10%) was found in the solution surrounding the posterior hindgut. The higher molecular weight galloylglucose esters of peaks C and D did not permeate the cuticle of the posterior hindgut.

DISCUSSION

Tannins mainly tend to adversely affect species that do not feed on plants containing large quantities of phenolics (Bernays, 1981). Terrestrial isopods are not confronted with high levels of phenolic compounds in their natural food, because many kinds of phenolics are removed from leaf litter by leaching (Kuiters and Sarink, 1986). In particular, gallotannins are present in overwintered litter during the following spring and summer at only low concentrations of 0.2–2.0 mg/g, while total phenolics can be found at concentrations of 1–10 mg/g (Zimmer, 1998). Thus, *P. scaber* can be expected not to have evolved specific mechanisms for counteracting potentially harmful effects of tannins.

Condensed tannins in leaf litter (2–5 mg/g) caused increased mortality of *P. scaber* (Zimmer and Topp, 1997a). Moreover, the development of juvenile *P. scaber* is affected by condensed tannins in the food (Zimmer, unpublished data).

Little is known about the effects of hydrolyzable tannins on isopods. Tannic acid is thought to be toxic to many invertebrates, but not antidigestive (Bernays, 1981). Alkalinity in the gut of many insects can reduce the protein-precipitating effects of tannins (Berenbaum, 1980; Martin and Martin, 1983) and, consequently, their digestibility-reducing features. Furthermore, surfactants in the gut of phytophages prevent tannin binding of digestive enzymes in vivo (Martin et al., 1987; DeVeau and Schultz, 1992). In terrestrial isopods, the gut content is slightly acidic, with a pH level of about 6 (Hartenstein, 1964; Zimmer and Topp, 1997b), but surfactants are present in *P. scaber* (Zimmer, 1997). Since gallic acid, as a product of gallotannin hydrolysis, may be harmless to some phytophagous insects (Bernays and Chamberlain, 1980; Hagerman and Butler, 1991), hydrolysis of gallotannins might serve as a mechanism of detoxification in isopods also.

Deleterious effects of phenolics may be initiated by oxidation (Appel, 1993). On the other hand, enzymatic oxidation of phenolic compounds is required for the degradation of lignin (Ander and Eriksson, 1976; Sinsabaugh and Linkins, 1987; Breznak and Brune, 1994), and this is important with respect to cellulose digestion by saprophagous animals (Ljungdahl and Eriksson, 1985; Breznak and Brune, 1994). Consequently, despite the potentially harmful effects of oxidation products, phenol oxidation can be expected in isopods.

Tannin–Microbiota Interactions. The RCR of *P. scaber* fed on artificial diet (0.20) was similar to that of adults of similar size feeding on poplar litter (0.25–0.34) (Wieser, 1965). Tannic acid and gallic acid significantly reduced the consumption rate of *P. scaber*. No determination was made as to whether this was the result of feeding deterrrence or some postingestive feedback mechanism. In some phytophagous insects, similar effects of hydrolyzable tannins on RCR have been observed (summarized in Mole and Waterman, 1987). In saprophagous invertebrates, leaching of phenolics increased consumption of litter (Cameron and LaPoint, 1978; Poinsot-Balaguer, 1993). In contrast to the present results on a saprophagous isopod, TA and GA stimulated feeding in several phytophagous species, although they deterred it in others (Bernays et al., 1980).

When *P. scaber* had been feeding on litter prior to the experiment, the observed digestibility did not show an influence of ingested tannic acid. However, after reducing microbial counts in the anterior hindgut by pre-experimental artificial diet, AD significantly decreased, indicating an important role of gut microbiota in digesting food (Hassall and Jennings, 1975; Reyes and Tiedje, 1976; Griffiths and Wood, 1985; Kukor and Martin, 1986). In contrast to tannic acid, gallic acid only slightly reduced, or even increased microbial counts in the gut and the hepatopancreas of *P. scaber*. Hence, antimicrobial effects of hydrolyzable tannins are mainly due to galloylglucose esters. Furthermore, ingested gallic acid increased digestibility, even if the woodlice had previously fed on antibiotics. Consequently, TA can be expected to exert stronger effects on saprophagous isopods than GA.

Hydrolysis of galloylglucose esters results in GA and glucose. Probably, these smaller compounds are drawn to the resorptive hepatopancreas together with fluids containing other products of digestion (Hames and Hopkin, 1989). By contrast, galloylglucose esters that may be more harmful than gallic acid are excluded from entering the hepatopancreas. Presumably, the hindgut cuticle serves as a filter in preventing the transport of these phenolics to the hepatopancreas (see below). While ingested tannic acid reduced the number of microorganisms in the hepatopancreas, GA led to increased microbial counts in the midgut lobes. Considering the potential importance of the hepatopancreatic bacteria in digestive processes (Zimmer and Topp, 1998a–c), excluding galloylglucose esters from entering the hepatopancreas leads to better conditions for food utilization. Consequently, the increase in the number of hepatopancreatic bacteria after ingesting GA appears to be correlated with the increased AD of the diet.

Compared to the results of other studies that have measured the fate of ingested gallotannins in insects, *P. scaber* is intermediate between insects that pass gallotannins through the gut unchanged (Barbehenn and Martin, 1992) and insects that extensively oxidize (Barbehenn and Martin, 1994) or hydrolyze gallotannins (Bernays and Chamberlain, 1980). Hydrolysis of hydrolyzable tannins occurred in the gut of *P. scaber* by microorganisms that could be partially eliminated by antibiotics. For the detoxification of tannins, extensive hydrolysis is required, resulting in nontoxic products, e.g., gallic acid and glucose (cf. Barbehenn et al., 1996). The number of platable (ingested) microorganisms in the hindgut was reduced by feeding on artificial diet in the experiment. Hence, under natural conditions, hydrolysis of gallotannins may be more extensive than was observed in the present study. In woodlice containing high numbers of gut microbiota derived from feeding on litter, hydrolysis of galloylglucose esters resulted in a high level of gallic acid in the anterior hindgut.

The net production of brown pigments in the feces when isopods were fed diet containing TA clearly indicates phenol oxidation in the gut of *P. scaber*. A reduction of platable microorganisms in the hindgut by feeding on artificial diet prior to the experiment did not change the amount of brown pigments in the feces. Thus, phenol oxidation does not seem to depend on ingested bacteria or fungi. By contrast, antibiotics in the diet that reduced the number of bacteria in the hepatopancreas resulted in strongly decreased phenol oxidation. Similar results were obtained from feeding experiments for the estimation of gallic acid digestion. After feeding on litter prior to the experiment, 15–35% of the ingested GA could be recovered from the feces. The difference was assumed to have been oxidized or absorbed. Similar values (15–25%) were obtained after feeding on artificial diet prior to the experiment. Presumably, the reduced number of hindgut microbiota did not influence the recovery of gallic acid. Ingested antibiotics led to 50–80% recovery of ingested GA. In this case, in addition to the platable

microorganisms in the hindgut, microbial counts of the hepatopancreas were reduced. These results suggest an involvement of the hepatopancreatic bacteria in phenol oxidation. By contrast, hydrolysis of gallotannins is mainly brought about by microorganisms ingested with the food.

The terrestrial isopod *Oniscus asellus* Linnaeus is capable of oxidatively metabolizing several phenolic compounds to CO_2 (Neuhauser and Hartenstein, 1976). The authors suggested that the necessary enzymatic activity is attributable to "the gut flora or other internal microbes, ... since complete catabolism of aromatics has been obtained only for microbes" (Neuhauser and Hartenstein, 1976). Zimmer and Topp (1998c) drew similar conclusions concerning the oxidation of phenolics by hepatopancreatic bacteria in *P. scaber*.

Protective Function of Hindgut Cuticle. In many insects, a membranous peritrophic envelope is supposed to assist digestive processes by allowing compartmentalization of the gut lumen (Terra et al., 1979; Santos and Terra, 1986). Possibly, in terrestrial isopods the hindgut cuticle and an ectocuticular space (Holdich and Mayes, 1975) that is formed by numerous "microvilli-like structures" of the epithelial cells (Lane, 1988) have similar functions. Given that the hindgut cuticle is selectively permeable to certain compounds, fluids containing the products of digestion will be compartmentalized from the gut lumen where digestion takes place, and nutrients can be transported to the resorptive regions of the hepatopancreas via the ectocuticular space or by absorbing nutrients into the epithelial cells of the anterior hindgut (cf. Lane, 1988) and directly passing nutrients to the hepatopancreas via the hemolymph. By contrast, Hames and Hopkin (1989) described gut fluids containing digested nutrients being drawn to the hepatopancreas via the typhlosole canals. In this case, water-soluble compounds of the food would be transported to the hepatopancreas. However, only gallic acid and peak B were detected in these midgut lobes, while none of the higher molecular weight galloylglucose esters was found there. These results are in accord with the permeability of the hindgut cuticle towards TA compounds. Obviously, the hindgut cuticle—besides allowing compartmentalization—serves as a protective filter in keeping galloylglucose esters inside the hindgut lumen, while exhibiting permeability to gallic acid. In some phytophagous insects, the peritrophic envelope serves as a barrier to tannic acid (Barbehenn and Martin, 1992, 1994). These authors proved that the peritrophic envelope of lepidopteran larvae was permeable to gallic acid, but nearly impermeable to galloylglucose esters (Barbehenn and Martin, 1992, 1994). However, the containment of gallotannins inside the gut lumen is not sufficient to protect tannin-sensitive species from the impact of tannins (Barbehenn and Martin, 1994). Gallic acid, which freely penetrated the hindgut cuticle of *P. scaber*, has beneficial effects on the growth of tree-feeding insects (Bernays et al., 1983) and probably is nontoxic to isopods. Toxic effects of galloylglucose esters are prevented by surfactants in the hindgut fluids of *P. scaber* (Zimmer, 1997).

CONCLUSIONS

The present results show that ingested galloylglucose esters are kept inside the endocuticular gut lumen. In contrast to galloylglucose esters, gallic acid penetrates the hindgut cuticle and is transported to the hepatopancreas, where it supports the maintenance of high population densities of hepatopancreatic bacteria involved in cellulose digestion (Zimmer and Topp, 1998a,b) and phenol oxidation (Zimmer and Topp, 1998c; this study). In the hindgut lumen, galloylglucose esters decrease the number of ingested microorganisms thought to be important in digestion (Hassall and Jennings, 1975; Reyes and Tiedje, 1976; Griffiths and Wood, 1985; Kukor and Martin, 1986) and hydrolysis of gallotannins (this study). In phytophagous insects, inhibitory effects on gut microbiota have been suspected to be the main reason for the impact of tannins (Martin et al., 1987). The present results suggest that similar mechanisms are important in terrestrial isopods.

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PHEROMONE SHARING: BLENDS BASED ON THE SAME COMPOUNDS FOR *Euschistus heros* AND *Piezodorus guildinii*

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Abstract—Pheromone compounds shared between two species of pentatomids from the soybean stink bug complex are reported. Two male-specific compounds (R_t 27.47 and 29.62 min) were detected in the airborne extract of *Piezodorus guildinii* by gas chromatography and mass spectrometry analysis. The compounds were identified as methyl 2,6,10-trimethyldodecanoate and methyl 2,6,10-trimethyltridecanoate.

Key Words—Soybean, methyl 2,6,10-trimethyldodecanoate, methyl 2,6,10-trimethyltridecanoate, stinkbugs, pheromones, *Piezodorus guildinii*.

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**In memoriam.

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INTRODUCTION

Soybean, *Glycine max* (L.) Merryl, is severely attacked by complexes of stink bugs around the world. In Brazil, the most important members of the complex are *Nezara viridula* (L.), *Piezodorus guildinii* (westwood), and *Euschistus heros* (F.) (Panizzi and Rossi, 1991). This attack has led to the use of more than four million liters of chemical insecticides to control these pests (Corrêa-Ferreira and Moscardi, 1996). The use of semiochemicals in biological control of these pests would be a more ecologically benign approach to their integrated pest management.

The first attractant pheromone identified for a member of the soybean stink bug complex was from the southern green stink bug, *N. viridula* (Baker et al., 1987; Aldrich et al., 1987). Field tests of synthetic blends have been conducted on a small scale, but very few bugs or tachinid parasites were attracted (Aldrich et al., 1993).

Recently, the chemical communication system of the Neotropical brown stink bug, *E. heros*, was elucidated during trapping studies of its Nearctic relative *E. obscurus* (Borges and Aldrich, 1994; Aldrich et al., 1994). The synthesis of a racemic mixture of methyl 2,6,10-trimethyltridecanoate and stereoisomeric mixture of methyl 2,6,10-trimethyldodecanoate, two components of the male-produced pheromone of *E. heros*, was conducted by Mori and Murata (1994) and Ferreira and Zarbin (1996), respectively. In spite of the identification and synthesis process, those compounds were never tested against *E. heros*. Recently, confirmation that methyl 2,6,10-trimethyltridecanoate is a male-produced pheromone of *E. heros* was presented by Borges et al. (1998a). In recent field tests we evaluated the response of stink bugs to natural (airborne extracts) and synthetic racemic mixtures of methyl 2,6,10-trimethyltridecanoate. The presence of stink bugs and their egg parasitoids was recorded in pheromone-baited traps. During these tests, *P. guildinii* was caught in the baited traps in significantly greater numbers than other insects, i.e., *P. guildinii* comprised 63% of the species present in the baited traps, followed by *E. heros* (10%) and *N. viridula* (10%). Other species, such as *Thyanta perditor*, *Acrosternum aseadum*, and *Edessa meditabunda*, comprised less than 5% of the catch (Borges et al., 1998b).

In this paper we report the identification of methyl 2,6,10-trimethyldodecanoate and methyl 2,6,10-trimethyltridecanoate in the airborne volatiles collected from *P. guildinii* males.

METHODS AND MATERIALS

Insects. P. guildinii adults were obtained from a soybean field (1 ha) at Cenargen (National Research Center for Genetic Resources and Biotechnology),

and their offspring were reared on sunflower seeds, soybeans, and green beans at 26 ± 0.5°C and 65 ± 10% relative humidity with a 14L : 10D photoperiod. To prevent olfactory interactions between the sexes, males were separated from females after the imaginal molt and cuticular hardening, but before sexual maturity.

Collection of Volatiles. Samples were collected from groups of 16 virgin adult male ($N = 80$ or female ($N = 48$) *P. guildinii* of known age by coaxing the bugs into a 500-ml glass column and trapping volatiles from the air drawn by vacuum (100 ml/min) over the bugs and through 30 mg of activated charcoal for 24 hr. Trapped volatiles were eluted from the filter with 150 μl of *n*-hexane and stored at −20°C (Borges and Aldrich, 1994).

GC-MS Analysis. GC-MS analyses were carried out on a Shimadzu QP-5000 GC-MS spectrometer linked to a TIC detector in splitless injector mode. The DB-5 capillary column (30 m × 0.53 mm × 0.25 μm was operated at 50°C for 1 min, increased to 150°C at a rate of 5°C/min, held at this temperature for 5 min, increased to 280°C at a rate of 10°C/min, and finally held at this temperature for 10 min.

RESULTS

Two male-specific compounds (R_t 27.47 and 29.62 min) were detected in the airborne extract of *P. guildinii*, corresponding to methyl 2,6,10-trimethyldodecanoate (**1**) (Figure 1A and B) and methyl 2,6,10-trimethyltridecanoate (**2**) (Figure 1A and C). The same compounds were previously observed in the pheromonal blend of the stink bug *E. heros* (Aldrich et al., 1994).

Identity of the two compounds was checked by coinjection with a synthetic sample prepared in our laboratory (Ferreira and Zarbin, 1996) and with one prepared by Mori and Murata (1994). Gas chromatograms of volatiles collected from female *P. guildinii* (not shown) totally lacked compounds **1** and **2**.

DISCUSSION

Our finding of methyl 2,6,10-trimethyldodecanoate and methyl 2,6,10-trimethyltridecanoate in volatiles collected from *P. guildinii* explains by *P. guildinii* was attracted to field traps (Borges et al., 1998b) containing synthetic methyl 2,6,10-trimethyltridecanoate during pheromones studies of *E. heros*. Both compounds are present in the pheromone of *E. heros*, and both are part of the *P. guildinii* communication system. Males of *P. guildinii* release them in a 1 : 3 ratio of methyl 2,6,10-trimethyldodecanoate–methyl 2,6,10-trimethyltridecanoate. We have no information on the stereochemical importance of the stereogenic centers in the molecules to attraction of *P. guildinii*.

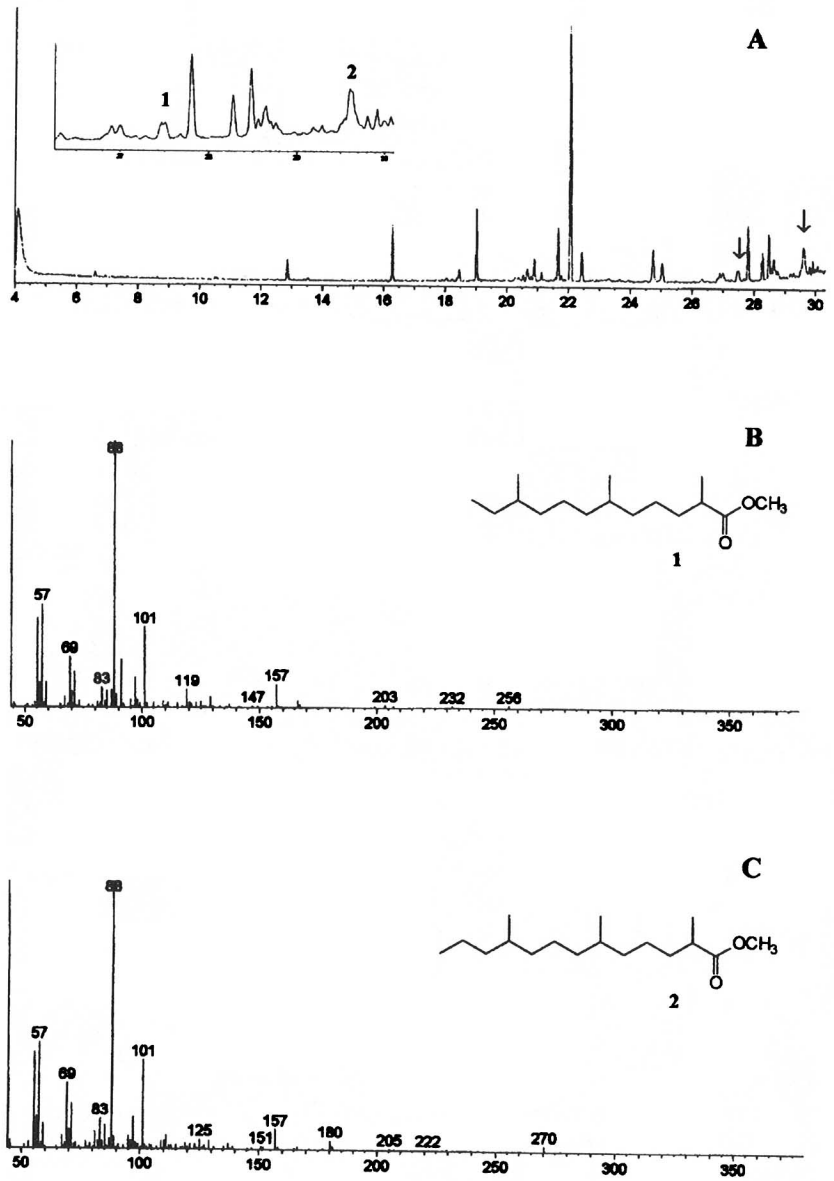

FIG. 1. GC-MS analysis of the airborne pheromone extract of *Piezodorus guildinii*. (A) chromatogram chart (arrows indicate the active compounds in the full chart. **1**: methyl 2,6,10-trimethyldodecanoate, and **2**: methyl 2,6,10-trimethyltridecanoate). (B) Mass spectrum of compound **1**. (C) mass spectrum of compound **2**.

Species-specific blends based on the same compounds among *Euschistus* species were reported by Aldrich et al. (1991). This phenomenon of pheromone sharing was also reported for *N. viridula* and the sister genus *Acrosternum* (Aldrich et al., 1993). Although the different species liberate a similar pheromone blend, unique ratios of these compounds might be involved in species isolation.

Synthetic methyl 2,6,10-trimethyltridecanoate is a very stable molecule (Prof. Kenji Mori, personal communication), and its stability may enable an integrated pest management program (IMP) to adopt another tool to control and monitor *E. heros* and *P. guildinii*. In addition, it may be possible to manipulate a stereoisomeric mixture containing methyl 2,6,10-trimethyl-tridecanoate to make a blend attractive for other species from the soybean stink bug complex.

Additional components from the *P. guildinii* pheromone blend remain to be identified. We believe, however, that these components have little or no influence in the long-distance communication behavior of this species because efficient attraction in the field is possible with the synthetic racemic mixture of methyl 2,6,10-trimethyltridecanoate (Borges et al., 1998b).

Pheromone blends based on the same compounds for two pentatomids species, *N. viridula* and species in the sister genus *Acrosternum*, were reported by Aldrich et al. (1993). In this work, we report the sharing of pheromone compounds between two species of the soybean stink bug complex in Brazil, *P. guildinii* and *E. heros*. Furthermore, we have now identified two male-released compounds from the sex pheromone of the stink bug, *P. guildinii*.

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THE DYNAMICS OF CARBON–NUTRIENT BALANCE: EFFECTS OF COTTONWOOD ACCLIMATION TO SHORT- AND LONG-TERM SHADE ON BEETLE FEEDING PREFERENCES

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Abstract—The carbon–nutrient balance hypothesis (CNBH) predicts that shading should increase leaf palatability to herbivores by decreasing concentrations of carbon (C) -based chemical defenses and increasing nitrogen (N). We measured cottonwood (*Populus deltoides*) growth, leaf chemistry, and beetle (*Plagiodera versicolora*) feeding preferences on saplings grown in either continuous high (HH) or low (LL) light, and saplings switched from high to low (HL) or low to high (LH) light for nine days. As expected, based on the CNBH, shading increased total N and decreased total phenol glycoside (C-based secondary metabolites) concentrations in plants from all shade treatments (LL, HL, and LH), relative to HH plants, with plant growth and gross leaf chemistry being affected by initial and final light regime. In contrast, while specific phenol glycoside concentrations were affected by the initial and final light regime, they also showed an initial × final light interaction. Beetles tended to prefer LL to HH plants. Beetles unexpectedly preferred HH to either HL or LH switched plants, most likely because high concentrations of a specific phenol glycoside – salicin – occurred in both switched treatments and inhibited beetle feeding. Plant chemical allocation during light acclimation led to unpredictable changes in specific C-based compounds, even though plant growth and gross chemistry conformed to expectations for shading effects and the CNBH. The response of this herbivore to altered concentrations of a specific compound confounded predictions based on average dynamics of suites of chemicals. Our findings may help explain why relationships between light

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availability and herbivory in field studies, where light varies on many time scales, can differ from those predicted by the CNBH. Understanding both dynamic plant chemical responses to altered resource availability and controls over allocation to specific compounds would likely enhance future predictability of specific environment-plant-herbivore interactions.

Key Words—Carbon–nutrient balance, shading, light acclimation, herbivory, phenol glycosides, salicin, *Plagiodera versicolora*, beetle feeding, eastern cottonwood, *Populus deltoides*.

INTRODUCTION

The carbon–nutrient balance hypothesis (CNBH) (Bryant et al., 1983) predicts that when plant carbon (C) availability is limited relative to nitrogen (N) (e.g., low light at high soil N), concentrations of foliar C-based defensive chemicals will decrease relative to the concentration of N and plants should be more palatable to herbivores. Conversely, when C availability is high relative to N (e.g., high light at low soil N), leaf N concentration should decrease, concentrations of C-based defensive chemicals should increase, and plants should be less palatable to herbivores.

Experimental studies on the effects of shading using fast-growing plants with C-based secondary metabolites generally support the CNBH. Several studies have shown that shading decreased concentrations of C-based compounds in plant foliage and inferred that this would increase palatability to herbivores (e.g., Waterman et al., 1984; Waring et al., 1985; Mole et al., 1988; Mole and Waterman, 1988; Dudt and Shure, 1994). Shade decreased the concentration of phenol glycosides in willow leaves (*Salix* spp.) and increased beetle feeding (Larsson et al., 1986). Bryant et al. (1987) found decreased phenolic concentrations and increased snowshoe hare herbivory on shaded twigs of fast-growing Alaskan paper birch saplings, but no effect of shade on slower-growing alder saplings. Boreal feltleaf willows also had lower phenolic levels and were preferred by snowshoe hares after shading (Bryant, 1987). Reichardt et al. (1991) found that only one of six carbon-based secondary metabolites in Alaskan balsam poplar decreased after shading. In this case, snowshoe hare feeding was higher on shaded than on unshaded foliage, but the difference was not statistically significant. Finally, *Lymantria dispar* consumption and growth was greater on shaded than on sun leaves of *Populus* seedlings, and sun leaves had higher phenol glycoside concentrations (Lindroth et al., 1993).

In all the above studies, shaded plants were grown either from seedlings or cuttings raised entirely in shade or were shaded for an entire growing season or more. These plants were probably fully acclimated to constant low light availability. In nature, however, light availability varies over many time scales. These

include short-term understory sun flecks, cloudy days in open habitats, shading during and between growing seasons in early succession, long-term shading after forest gap closure, and increased light after gap formation. The responses of herbivores to shade and sun plants in field conditions are less consistent than those found with plants from controlled light experiments, as might be expected given greater variability in the spatial and temporal scale of light environments. Many field studies do show preference for and/or increased herbivory on shade plants (Louda and Rodman, 1983; Lincoln and Mooney, 1984; Basset, 1991; Peterson and Smitley, 1991; Dudt and Shure, 1994; Folgarait et al., 1995), but some show preference for and/or increased herbivory on sun plants (Nichols-Orians, 1991; Lowman, 1992). Some studies have found no significant effect of light environment (Harrison, 1987; Collinge and Louda, 1988; Aide and Zimmerman, 1990), and others show both increased and decreased preference and/or herbivory, depending on plant species and/or experimental design (Maiorana, 1981; Louda et al., 1987). Light-induced physiological and biochemical changes in plants may occur over days to weeks, depending on the rates of various acclimation processes [e.g., chemical allocation, (Reichardt et al., 1991) or rate of initiation of new leaves with novel morphology and/or physiology (Sims and Pearcy, 1991)]. During acclimation, plant characteristics influencing resistance to herbivores may well differ from those found once the plant is at equilibrium with the new light regime. These differences could potentially account for some of the discrepancies between predictions of the CNBH and field observations.

We compared the effects of short-term transitions between high and low light environments to the effects of long-term shade on eastern cottonwood (*Populus deltoides* L. Bartr.; Salicaceae) growth, leaf chemistry, and the feeding preference of a leaf-eating beetle (*Plagiodera versicolora* Laich; Chrysomelidae). Cottonwood is a resource-demanding, fast-growing, indeterminate species found primarily in high light and nutrient environments (Hosner and Minckler, 1963). Phenol glycosides are the primary class of C-based secondary metabolites in cottonwood (Lindroth et al., 1987; Bingaman and Hart, 1993; Picard et al., 1994). Concentrations of these compounds have been shown to change with altered light availability in other *Populus* and related *Salix* species (Waring et al., 1985; Larsson et al., 1986; Bryant et al., 1987; Reichardt et al., 1991). Feeding preference, consumption, and performance of *P. versicolora* on cottonwood have been shown to be affected by changes in phenol glycoside concentrations associated with stress and damage to cottonwood plants (Coleman and Jones, 1988; Jones and Coleman, 1988; Jones et al., 1993, 1994). In particular, high concentrations of one phenol glycoside, salicin, have been shown to inhibit feeding (Tahvanainen et al., 1985; Wait et al., 1998).

Based on the CNBH, we expected that long-term shade would result in reduced growth, plant C gain and phenol glycoside concentrations, increased leaf N concentration, and increased beetle feeding relative to plants from long-term

high light environments. We expected that plants switched from sun to shade or shade to sun would have intermediate growth and chemical characteristics and that beetles would prefer switched plants to those grown continuously in high light.

METHODS AND MATERIALS

Light Treatments and Plant Maintenance. Rooted shoot-cuttings of cottonwood clone ST 109 were grown at 85% full sun (ambient greenhouse light) until they had reached the three- to five-leaf stage, at approximately 20 days. Half the plants were then randomly selected for transfer to a large shade tent in a greenhouse, which reduced light to 28% full sun; half remained in ambient greenhouse light. Light levels were determined by monitoring light levels in each environment throughout the experiment by using a Li-Cor PAR sensor and regressing total daily insolation in each treatment against insolation at a nearby climate station (our estimate of full sun). Based on measurements of midday gas exchange rates in this cottonwood clone (Wait, 1992; Crone and Jones, unpublished data), 28% full sun was well below cottonwood light saturation levels of photosynthesis that occurs at around 750 μmol photons/m^2/sec (mean $\pm$ SD midday insolation was: 372 $\pm$ 175 μmol/m^2/sec), and 85% full sun treatments exceeded photosynthetic saturation on sunny days (mean $\pm$ SD midday insolation was: 894 $\pm$ 354 μmol/m^2/sec). Although greenhouse space prohibited using individual plant-replicated shade tents, sun and shade environments were on adjacent benches, separated sufficiently to avoid shading of high light plants. During the first month of the experiment, before plants were switched between treatments, saplings size showed no significant bench position effects (ANOVA, $N = 30$, $P > 0.10$), indicating that the effects of shade treatments were large relative to any potential effects of the position of treatments. Plants were grown in large pots filled with potting soil–vermiculite mixture and were regularly watered and fertilized (Jones and Coleman, 1988). Plant propagation began in April, and experiments were completed by early July.

After 40 days, 14 plants from each light treatment were randomly selected for continuous growth and leaf development measurements, beetle feeding preference assays, leaf chemical assays, and end-point harvest data. These plants were unbranched, single-leader, 15- to 25-leaf saplings that showed no visible signs of insect damage or disease. Of the 14 plants from the shade tent, half were randomly selected for transfer to sun (LH), while half remained in shade (LL). Of the high light plants, half were randomly selected for transfer to the shade tent (HL) and half remained in high light (HH). Feeding preference assays took place nine days after HL and LH plants were switched between light treatments. We will refer to the nine-day period between transfer and harvest as the

switch period of the experiment. With the exception of the three to five small leaves from the 20-day establishment period, most of which had senesced by the time plants were switched between environments, all leaves on HH and LL plants initiated and developed in high or low light, respectively. For HL and LH plants, the first leaf initiated in the new light environment reached the position of the first fully expanded leaf [Leaf Plastochron Index (LPI) -5 (Coleman and Jones, 1988)] by the end of the nine-day switch. Leaf position was determined using the LPI (Larson and Isebrands, 1971), where the first apical leaf > 2 cm in length with fully unfurled leaf margins was designated LPI-0, and leaves were subsequently numbered down the plant. LPI-5 was the leaf developmental stage used for all chemical and feeding preference assays.

Plant Growth Measurements. Plant height from the base of new stem growth to the apical bud meristem was measured daily on all plants during the week prior to switching plants between treatments and throughout the nine-day switch period. Relative height growth rates of each of the seven plants in each treatment were calculated from the average of daily difference in natural log-transformed height during the switch period.

The length and width of each leaf on each plant was measured on the first day of the switch period, and the length and width of all leaves that had not yet reached full expansion were measured daily throughout the switch period. Leaf initiation and abscission were recorded during daily measurements of leaf area. New leaves were counted when they reached LPI-0, and the position (LPI) of all expanding leaves was recorded and used to determine the position at which leaves reached full expansion. At the end of the experiment, leaf area of all leaves on all plants was measured (Li-Cor model 3000 leaf area meter). Changes in leaf area due to initiation, expansion, and abscission during the switch period were estimated from measured length and width using independent linear regressions for each treatment, based on harvest data from eight additional plants per treatment ($R^2 > 0.98, P < 0.001$ for all regressions). Relative leaf area increment rates were estimated from the average of daily difference in natural log-transformed area.

Plants were harvested on the day on which feeding trials were initiated, after switched plants had spent nine full days in the second environment. Plant height, stem diameter below the petiole of the leaf in position LPI-5 at the time of switching, number of leaves, leaf area, and leaf and stem fresh weight (FW) were recorded. Leaf, stem, and root dry weight (DW) were measured after oven drying.

Leaf Chemical Characteristics. At harvest, LPI-5 was excised from the plant at the tip of the petiole. As soon as leaf discs for bioassays (see below) were punched from leaves, the remaining portion of each leaf was immediately flash frozen in liquid nitrogen. Leaves were stored at −70°C (Revco Ultra-low freezer) until chemical analysis. Half of each leaf was analyzed for protein nitro-

gen using the modified Bradford assay of Jones et al. (1989), with RuBP as a calibration standard. Because chemical bioassays used fresh leaf material, concentrations are reported on a fresh weight basis.

The remaining half of each flash-frozen leaf was used to determine phenol glycoside (PG) content using an extraction and HPLC method modified from Lindroth and Pajutee (1987). Salicin, salicortin, tremuloidin, and tremulacin were identified in clone ST 109, based on coinjection with authentic standards obtained from T. Clausen and by comparison of the UV absorption spectra of peaks with stop-flow spectral scanning. Salicin, salicortin, and tremuloidin have been consistently reported to occur in *P. deltoides* (Lindroth et al., 1987; Bingaman and Hart, 1993; Picard et al., 1994). While tremulacin did not occur in a *P. deltoides* population from Wisconsin (Lindroth et al., 1987), it has now been found to occur in many, but not all, clones and populations of *P. deltoides* (Bingaman and Hart, 1993; Picard et al., 1994).

Frozen leaf material (−70°C) was rapidly homogenized (10 sec) in ice-cold methanol, immediately filtered (0.22 μm) and then stored at −15°C. The potential for changes in PG composition and concentration during extraction (salicortin is particularly susceptible to degradation to salicin) (Lindroth and Pajutee, 1987; Orians, 1995) was evaluated beforehand using test samples of LPI-5 from 10 plants of the same developmental stage grown under unshaded greenhouse conditions. Immediately following excision at the tip of the petiole, leaves were longitudinally split in half on either side of the mid-vein. One half was immediately cold-extracted in methanol, while the other half was flash-frozen and then cold-extracted in methanol (N = 10 leaves). Known amounts of the four phenol glycosides (PGs), approximating those found in leaves, were added to each leaf half prior to extraction of five leaves, while the remaining five leaves had no compounds added. Recovery of all added PGs was >95% for all test samples. Measured concentrations of added PGs did not differ from that expected, based on the amounts added, for either extraction method. PG concentrations in samples free from added PGs did not significantly differ between extraction methods. Measured amounts of PGs in leaf samples free from added PGs and extracted by either method, did not significantly differ from that found in samples to which PGs had been added, after subtracting the known amount of PGs added. Periodic, repeated HPLC analyses of extracts from flash-frozen leaves stored at −15°C showed that PG composition and concentrations were stable for at least three months, although all samples were analyzed well within this time period.

A trace amount of resorcinol was added to the leaf material immediately prior to homogenization in order to estimate recovery in each sample. Resorcinol recovery was always >95%. 2,4-Dimethoxy benzene (DMB) was added to extracts as an external standard. Samples were analyzed by reverse-phase HPLC (250 × 4.6 mm Alltech Absorbosphere HS C18, 5 μm; on a Varian 5500), using a

methanol/water + tetrohydrofuran gradient, and a UV detector at 254 nm. Peaks in each sample were identified by retention time and relative retention time compared to resorcinol and DMB. Concentrations, corrected by percentage recovery of resorcinol, were calculated from average peak areas of two replicate injections per sample. If replicate injections differed by more than ±15%, samples were run again, and the two closest values averaged. Total phenol glycoside (TPG) concentration was calculated by summing the concentrations of the four compounds. Concentrations are reported on a fresh weight basis.

Leaf total carbon (C) and total nitrogen (N) were estimated by measuring the total C and N of the methanolic extract. This approximation of the percentage of C and N in intact leaves was made necessary by limitations in leaf material imposed by our desire to measure all chemical characteristics and beetle feeding preference on the same leaf. The technique also had the advantage of distinguishing readily extractable C and N pools from structural materials (Jones and Coleman, 1989). Methanol extracts were evaporated to dryness and the percentages of C and N were determined on a Carlo-Erba NA 1500 CNS analyzer, using the average of triplicate analytical aliquots, and acetanilide as a calibration standard. We estimated LPI-5 leaf water content from the ratio of the combined dry weights of extraction pellets from MeOH and protein extractions to the fresh weight of the material used in these extractions. For consistency with other chemical parameters, we used water content estimates to convert the percentages of C and N to a fresh weight basis.

Beetle Feeding Preference Assays. *P. versicolora* feeds on several plant species in the Salicaceae, including willow and cottonwood (Wade and Bredan, 1986), and was locally abundant. Insects used in the experiments were field collected from *Salix* spp. 24-48 hr before feeding trials and maintained in the laboratory on field-collected willow leaves. Insects were not fed on cottonwood prior to experiments in order to minimize any potentially confounding effects of prior experience of cottonwood leaves grown in a particular light environment. Preference was determined in three separate dual-choice assays: HH vs. LL, HH vs. HL, and HH vs. LH, using a design based on Jones et al. (1993). Seven sets of four plants, one from each treatment, were matched prior to switches between light levels, based on similarity of size and relative height growth rates. For each set, a single high light plant was paired with a plant from either LL, HL, or LH treatments in a separate feeding arena.

Leaf discs (17 mm diameter) were punched from the intervein areas of the leaf in position LPI-5 on each of the seven plants per treatment (N = 7 replicates). Five discs from a single shade (LL, LH, or HL) plant and five discs from the paired HH plant were placed in a stratified array in an enclosed plastic arena (12 × 18 cm) lined with filter paper and moistened with deionized water. Thirteen adult beetles (mixed sexes) were added to each arena. Arenas were placed in a growth chamber at 22°C day, 18°C night, 16-hr photoperiod and >75% rel-

ative humidity. The area of each disc consumed was visually estimated to the nearest 5% every 4 hr the first 12 hr, and every 12 hr for the next 84 hr. Leaf area estimates were checked against a square-millimeter grid at the end of the experiment and were within 10% of measured areas. For each time period, the total consumption on all discs from a leaf of each treatment in an arena (= plant replicate) was summed.

Statistical Analyses. Plant size, growth rate, and gross and specific leaf chemical data were analyzed to answer three questions: (1) Were overall changes in these variables and parameters affected by light availability? (2) Were the directions and patterns of change consistent with classical and CNBH expectations for changes in growth, morphology, and chemistry? (3) Were the characteristics of switched plants intermediate? We therefore analyzed suites of variables (size at harvest, growth rates, gross leaf chemistry and specific leaf PG concentrations; see Table 1 below for list) using multivariate analysis of variance (MANOVA), followed by univariate analyses comparing individual variables included in the MANOVA (Barker and Barker, 1984; Scheiner, 1993) (see Tables 2 and 3 below for lists). The MANOVA included terms for the initial light treatment, final light treatment, which was the light regime during the switch period, and initial × final light interaction. The interaction term identified response variables for which the characteristics of switched plants (HL and LH) differed significantly from a linear additive combination of the effects of initial and final light availability.

Based on classical studies of the effects of light on plant morphology (e.g., Grime, 1966; Larcher, 1980; Smith, 1981), we expected that, in addition to decreasing overall plant size and growth rates, shade would decrease root–shoot ratios, decrease leaf specific mass, and increase leaf water content. To elucidate the contributions of these shifts to the overall MANOVA results, we included univariate tests of these variables.

As with size, growth rate, and gross leaf chemical characteristics variables, we also calculated univariate statistics for two additional gross leaf chemical variables that we expected to change with light, based on the CNBH: the C/N ratio and the PG allocation as the ratio of total phenol glycosides to total C (i.e., the proportional allocation of carbon to phenol glycosides). Gross leaf chemistry and concentrations of specific phenol glycosides were analyzed in separate MANOVAs. All calculations were performed using SYSTAT data analysis software.

Feeding preference was estimated as follows: For each replicate plant pair, the summed consumption on high light (HH) and shaded (LL, HL, or LH) leaf discs in each arena at each time point were the raw data used. The consumption values over the 100-hr period were used to derive an overall consumption rate for each treatment in each arena using linear regression. Because feeding preference assays must measure consumption on a given treatment relative to

another treatment, and cannot therefore be considered independent, statistical comparisons were based on the difference in consumption rates within each arena (Jones et al., 1993). Paired t tests within treatments tested whether the difference in consumption rates were significantly different from zero (one-tailed HH vs. LL, based on prior expectations of the CNBH; otherwise two-tailed), and the ANOVA of differences in consumption rates compared the direction of responses across treatments. In order to test the sensitivity of statistical inference for this analytical method, we also carried out analyses comparing means of absolute consumption at arbitrary fixed times of the analyses during the assay, repeated measures ANOVA, and nonlinear analysis of preference indices (ranking based on which treatment was consumed more). All these different analyses gave the same results, but we only report the results of consumption rate analysis, based on the advice of statisticians that this procedure is the least subject to bias.

Relationships between leaf chemical characteristics and beetle feeding preferences were analyzed using stepwise additive multiple linear regression ($P = 0.15$ inclusion criterion). Beetle feeding preference, as the difference in consumption rates between HH and other treatments within an arena (LL, LH, or HL), was regressed against differences in leaf gross and specific chemical characteristics for the same pair of leaves in the arena, across all shade treatments. Variables included are listed in Table 2c and d and Table 3c and d below. Data were expressed by plotting observed differences in consumption rates against predicted differences in consumption rates from the stepwise additive regression. Identical results were obtained in a stepwise subtractive multiple linear regression that began with all variables and sequentially removed the least significant terms.

RESULTS

Plant Size and Growth Rates. The effects of light on plant size at harvest arose from the additive effects of initial and final light environments. Both initial and final light contributed significantly to these relationships, but there was no significant interaction between initial and final light (Table 1). The HH plants had greater stem and leaf weights, larger stem diameters, higher leaf specific mass, and lower water content than LL plants, while HL and LH plants generally had intermediate values (Table 2a). The same pattern of increased plant height and number of leaves at high light was observed, although these variables did not significantly differ across light treatments (Table 2a). Differences in plant size measured at harvest reflected significant differences in initial light effects for all variables except leaf area and root/shoot ratio, together with final light effects for stem and leaf dry weights (Table 3a). Differences in root/shoot ratio were not statistically different (Tables 2a and 3a), but the trend was in the expected direction of higher allocation to roots in HH plants.

TABLE 1. MANOVA FOR EFFECTS OF LIGHT TREATMENT ON PLANT SIZE, GROWTH RATE, GROSS LEAF CHEMISTRY, AND SPECIFIC PHENOL GLYCOSIDE (PG) CONCENTRATIONS OF LPI-5[a]

		Initial light effect			Final light effect			Initial × final light		
Variable	*df*	Pillai trace	*F*	*P*	Pillai trace	*F*	*P*	Pillai trace	*F*	*P*
Plant size at harvest	9,16	0.907	17.381	<0.001	0.869	11.825	<0.001	0.464	1.538	0.217
Plant growth rate	4,21	0.651	9.799	<0.001	0.062	0.345	0.844	0.190	1.231	0.328
LPI-5 gross Leaf chemistry	5,20	0.691	8.954	<0.001	0.850	22.709	<0.001	0.425	1.295	0.305
LPI-5 specific PG concentrations	4,21	0.679	11.089	<0.001	0.706	12.591	<0.001	0.669	10.629	<0.001

[a]For list of specific variables see Table 2.

TABLE 2. VARIABLES INCLUDED IN MANOVA FOR EACH LIGHT TREATMENT[a]

Trait measured	Light treatment			
	HH	HL	LH	LL
a. Plant size at harvest				
Height (cm)	74.6 ± 3.1	75.9 ± 4.2	60.5 ± 6.0	58.3 ± 6.2
Number of leaves	18.3 ± 0.6	18.6 ± 0.8	17.0 ± 0.6	16.6 ± 0.5
Stem diameter (mm)	6.8 ± 0.2a	6.6 ± 0.1a	5.7 ± 0.4b	5.5 ± 0.3b
Stem fresh weight (g)	24.1 ± 1.4a	23.3 ± 1.6a	17.7 ± 2.4ab	15.2 ± 2.3b
Leaf fresh weight (g)	35.6 ± 1.8a	36.2 ± 2.4a	28.5 ± 2.8ab	25.8 ± 2.8b
Root/shoot dry mass ratio*	0.488 ± 0.037	0.468 ± 0.025	0.438 ± 0.055	0.463 ± 0.038
Leaf specific mass (mg/cm^2)*	5.78 ± 0.17a	4.78 ± 0.13b	4.61 ± 0.13b	3.74 ± 0.15c
Leaf water content (% FW)*	72.6 ± 0.5a	76.6 ± 0.3b	74.1 ± 0.6a	77.9 ± 0.6b
b. Plant growth rates				
Relative height growth rate (cm/cm/day)	0.024 ± 0.002ab	0.026 ± 0.001a	0.023 ± 0.002ab	0.018 ± 0.002b
Relative leaf area increment rate ($cm^2/cm^2/day$)	0.023 ± 0.004	0.026 ± 0.002	0.035 ± 0.005	0.024 ± 0.004
Number of new leaves initiated	3.7 ± 0.2	4.0 ± 0.3	3.3 ± 0.2	3.1 ± 0.4
Number of leaves abscised	2.9 ± 0.7a	2.4 ± 0.5a	0.6 ± 0.2b	1.1 ± 0.3ab
c. LPI-5 gross leaf chemistry				
Total C (extractable, % FW)	4.38 ± 0.03a	4.29 ± 0.04a	4.92 ± 0.04a	4.05 ± 0.11b
Total N (extractable, % FW)	0.386 ± 0.027a	0.638 ± 0.019b	0.425 ± 0.026a	0.563 ± 0.023b
H_2O (% FW)	89.8 ± 0.5ab	90.8 ± 0.3a	87.9 ± 0.9b	89.3 ± 0.4ab
TPG concentration (mg/g FW)	66.94 ± 4.41a	45.68 ± 4.80b	38.92 ± 4.45b	29.35 ± 4.65b
Protein (extractable, mg RuBP eq/g FW)	57.6 ± 6.2	61.7 ± 4.9	76.2 ± 6.7	59.1 ± 3.5
C/N ratio*	11.63 ± 0.66a	6.76 ± 0.17b	10.30 ± 0.57a	7.28 ± 0.40b
PG allocation (TPG/C)*	1.53 ± 0.10a	1.07 ± 0.11b	0.91 ± 0.11b	0.74 ± 0.13b
d. LPI-5 specific PG concentrations				
Salicin (mg/g FW)	3.76 ± 0.72a	8.60 ± 0.72c	6.88 ± 0.54bc	5.39 ± 0.42ab
Salicortin (mg/g FW)	12.96 ± 1.16	9.25 ± 2.62	6.90 ± 1.07	7.20 ± 2.26
Tremuloidin (mg/g FW)	21.49 ± 2.08a	16.19 ± 1.34ab	14.04 ± 2.12bc	8.76 ± 1.71c
Tremulacin (mg/g FW)	28.74 ± 2.20a	11.65 ± 1.33b	11.10 ± 1.47b	8.02 ± 1.19b

[a]Values are means ± SE. HH = constant high light; HL = switched from high to low light; LH = switched from low to high light; LL = constant low light). Means that differed significantly by Tukey's post hoc comparison of means (following univariate F tests; see Table 3) are indicated by different letters. *Indicates derived variables that were not included as raw inputs to the MANOVA, but were analyzed in univariate tests (see Methods and Materials).

TABLE 3. UNIVARIATE HYPOTHESIS TEST STATISTICS FOR VARIABLES INCLUDED IN MANOVA AND DERIVED VARIABLES WITH *a Priori* HYPOTHESES ABOUT EFFECTS OF LIGHT ENVIRONMENT

		Initial light effect		Final light effect		Initial × final light	
Trait measured	*df*	*F*	*P*	*F*	*P*	*F*	*P*
a. Plant size at harvest							
Height (cm)	1,24	5.880	0.023	0.167	0.686	0.001	0.972
Number of leaves	1,24	6.991	0.014	0.013	0.909	0.330	0.571
Stem diameter (mm)	1,24	21.190	<0.001	0.553	0.464	0.013	0.910
Stem fresh weight (g)	1,24	13.882	0.001	0.748	0.396	0.213	0.649
Leaf fresh weight (g)	1,24	12.312	0.002	0.180	0.676	0.478	0.496
Leaf area (cm^2)	1,24	1.979	0.172	0.001	0.981	0.459	0.504
Stem dry weight (g)	1,24	15.223	0.001	4.069	0.055	0.118	0.734
Leaf dry weight (g)	1,24	12.117	0.002	4.344	0.048	0.076	0.785
Root dry weight (g)	1,24	17.636	<0.001	3.297	0.082	0.217	0.646
Root/shoot dry mass ratio*	1,24	0.463	0.503	0.005	0.942	0.310	0.583
Leaf specific mass (mg/cm^2)*	1,24	57.866	<0.001	41.789	<0.001	0.196	0.662
Leaf water content (% FW)*	1,24	6.900	0.015	56.061	<0.001	0.026	0.873

b. Plant growth rates							
Relative height growth rate (cm/cm/day)	1,24	7.300	0.012	1.016	0.323	2.841	0.105
Relative leaf area increment rate (cm^2/cm^2/day)	1,24	3.996	0.057	0.368	0.550	1.635	0.213
Number of new leaves initiated	1,24	4.464	0.045	0.280	0.602	0.279	0.602
Number of leaves abscised	1,24	16.162	0.001	0.026	0.874	1.267	0.271
c. LPI-5 gross leaf chemistry							
Total C (extractable, % FW)	1,24	7.503	0.011	7.658	0.011	1.479	0.236
Total N (extractable, % FW)	1,24	0.560	0.461	66.241	<0.001	5.634	0.026
H_2O (% FW)	1,24	8.952	0.006	4.448	0.046	0.199	0.659
Protein (extractable, mg RuBP eq/g FW)	1,24	2.126	0.158	1.412	0.246	3.750	0.065
TPG concentration (mg/g FW)	1,24	23.446	<0.001	11.318	0.003	1.628	0.214
C/N ratio*	1,24	0.679	0.418	66.055	<0.001	3.640	0.068
PG allocation (PG/C)*	1,24	17.570	<0.001	7.884	0.010	1.680	0.207
d. LPI-5 specific PG concentrations							
Salicin (mg/g FW)	1,24	0.005	0.947	7.425	0.012	26.620	<0.001
Salicortin (mg/g FW)	1,24	4.545	0.043	0.810	0.377	1.103	0.304
Tremuloidin (mg/g FW)	1,24	16.360	<0.001	8.269	0.008	0.000	0.997
Tremulacin (mg/g FW)	1,24	44.523	<0.001	39.952	<0.001	19.289	<0.001

*Indicates derived variables that were not included as raw inputs to the MANOVA, but were analyzed in univariate tests (see Methods and Materials).

Plant growth rate variables were also affected by light availability. However, only the initial light environment explained a significant amount of variation in growth rate variables (Table 1). This result reflected the tendency for HH and HL plants to have higher height growth rates and lower leaf abscission rates than LH and LL plants (Table 2b). The nonsignificant effect of final light on plant growth rate (Table 1) appears to be somewhat counterintuitive at first, because long-term plant growth in HH was clearly greater than plant growth in LL (HH plants were larger than LL plants, Table 2a), indicating that growth was light-limited. We presume that short-term etiolation in response to lower light was occurring, and HL and LH plant growth rates would eventually have equilibrated to the new light environments if the experiments had run for a longer time period. Nevertheless, over this time period there were no significant effects of final light nor any significant interaction between initial and final light for any of the plant growth rate variables (Table 3b).

Leaf Chemistry. As with plant size at harvest, LPI-5 gross leaf chemistry [total C, total N, H_2O, protein, total phenol glycosides (TPG), C/N ratio and PG allocation] could be explained primarily from the additive effects of both initial and final light treatments with no significant initial × final light interactions (Table 1). However, in contrast to the plant size at harvest variables, which changed approximately in accord with the overall amount of light received in the entire experiment, chemical variables within this group reflected either initial or final light conditions, with only leaf total N showing any significant initial × final light interaction (Table 3c). For example, leaf total N depended primarily on the light environment in which leaves developed, being lower in HH and LH than in HL and LL plants. Leaf total C of LL plants was significantly lower than that of plants from any high light treatment (HH, HL, and LH), while TPGs were higher in HH plants than in plants from any of the low light treatments (HL, LH, and LL) (Table 2c). Interestingly, leaf water content was highest in HL plants, lowest in LH plants, and intermediate in HH and LL plants (Table 2c). In general, intermediate treatments tended to have intermediate values for gross chemistry, irrespective of significant differences (e.g., TPGs, Table 2c). In accordance with the CNBH, leaves that developed in high light (HH and LH) had substantially higher C/N ratios than leaves from low-light environments (LL and HL). Furthermore, the proportion of C allocated to phenol glycosides was significantly lower in plants from all shade treatments than in HH plants (Table 2c).

Specific phenol glycoside concentrations as a group variable also showed significant initial and final light effects. However, in marked contrast to gross leaf chemistry, as well as plant size and growth rates, this group variable was the only one to show a significant initial × final light interaction (Table 1). Within this specific PG group, salicortin was affected only by initial light; tremuloidin was affected by initial and final light; tremulacin showed a significant initial × final light interaction and was also affected by initial and final light; while

salicin showed a significant initial × final light interaction and a final light effect (Table 3d). In other words, each specific compound showed a different pattern of light treatment effects. Of the four individual compounds, three (salicortin, tremuloidin, and tremulacin) showed allocation patterns more or less in accord with the CNBH. Concentrations of each of these three compounds were generally highest in HH treatments, lowest in LL treatments, and intermediate in switched plants, although the patterns were not always statistically significant (Table 2d). The same could not be said for salicin, however, because concentrations were significantly highest in switched plants (Table 2d).

Beetle Feeding Preferences. Beetle feeding preference was not a simple consequence of the additive combination of short- and long-term light availability. Beetles tended to prefer LL to HH foliage, as expected based on the CNBH, although this result was not statistically significant at the $P = 0.05$ level ($P = 0.064$, Table 4, Figure 1A). In contrast, beetles showed an unexpectedly strong significant preference for HH over LH leaves (Table 4, Figure 1B), as opposed to either preferring previously shaded leaves or showing no preference. Beetles also tended to prefer HH over HL foliage, although this pattern was not statistically significant (Table 4, Figure 1C). The overall effects of HL and LH plants on herbivore preference for HH foliage were not statistically indistinguishable from each other, but both switched treatments did differ statistically from the preference tendency for shaded plants exhibited by beetles in the HH vs. LL trial (Table 4).

Beetle Preference and Leaf Chemistry. Of all the gross and specific leaf chemical variables (Table 2c and d), only protein and salicin concentrations

TABLE 4. BEETLE FEEDING PREFERENCE BASED ON DIFFERENCES IN CONSUMPTION RATES ON DISCS FROM HIGH LIGHT (HH) AND LOW LIGHT PLANTS (LL, LH, HL)[a]

Low light treatment	Mean difference (mm^2/h; HH–low light)	t	df	P
LL	−0.674	−1.761	6	0.064[b]
LH	0.993	3.780	6	0.009
HL	0.544	1.233	6	0.264
ANOVA (effect = shade treatment)				
$F_{2,18} = 5.437$; $P = 0.014$; rank order[c] $LL^a > LH^b \geq HL^b$.				

[a]Negative numbers indicate preference for low light foliage (LL, LH or HL), while positive numbers indicate preference for high light (HH) foliage. Paired t tests are for the null hypothesis that low light had no effect on feeding rates (mean different = 0) for a treatment relative to high light for each feeding arena. ANOVA was then used to test the null hypothesis that preference was independent of low light treatment (LL, LH or HL). See Methods and Materials for details.

[b]P-value is based on 1-tailed test (see Methods and Materials).

[c]Significance groups (Fisher's LSD) are indicated by lettered superscripts.

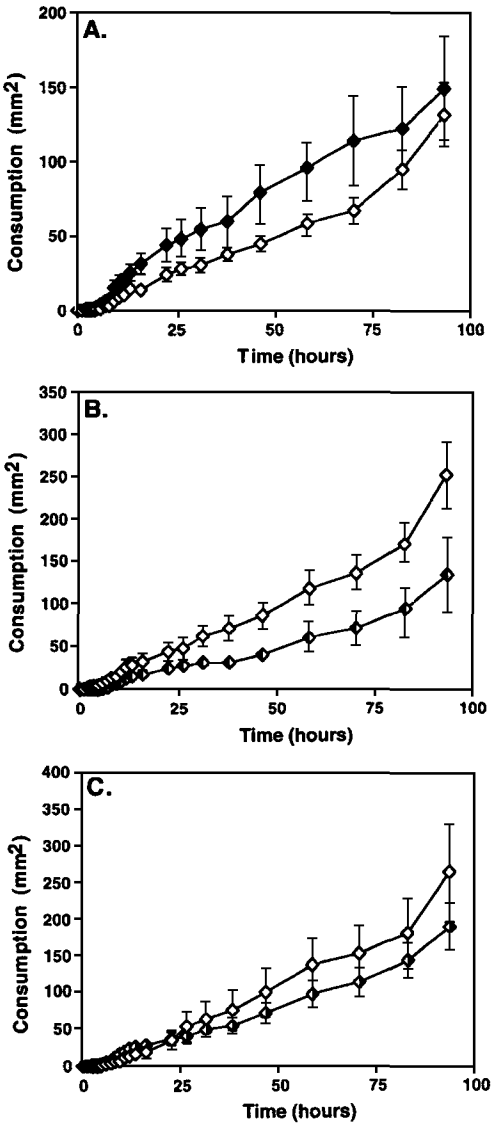

FIG. 1. Patterns of beetle total consumption over time (mean ± standard error, $N = 7$ arenas per assay) in dual choice assays comparing high light plants to other treatments. (A) High light (HH, ◇) versus low light (LL, ◆). (B) High light versus plants switched from low to high light (LH, ◇). (C) High light versus plants switched from high to low light (HL, ◇). For statistical analyses see Table 4 and Methods and Materials.

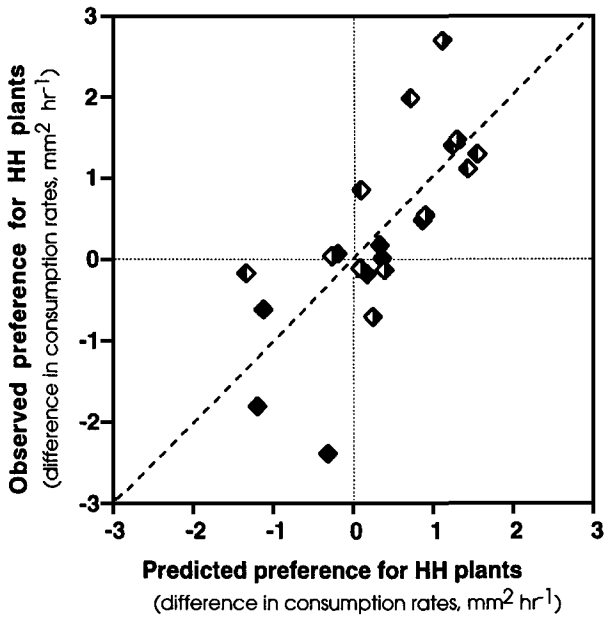

FIG. 2. Observed versus predicted beetle feeding preference for high light (HH) plants compared to low light plants (LL, ♦); plants switched from low to high light (LH, ◇); and plants switched from high to low light (HL, ◇). The observed difference in beetle consumption rates within each arena (N = 7 arenas in each of three assays) is plotted against the predicted beetle feeding preference (predicted difference in consumption rates; N = 7 arenas in each of three assays) from a stepwise linear multiple regression based on differences in chemical composition (HH − LL, HH − LH, or HH − HL) of leaves of each plant pair used in each assay. Only protein and salicin concentrations significantly entered the model (see Results and Methods and Materials for details). The dotted line indicates perfect correspondence (observed = predicted). Positive values show preference for HH plants over other treatments. Negative values show preference for LL or switched (HL, LH) plants over HH plants.

significantly entered the stepwise multiple regression for differences in beetle feeding versus differences in leaf chemistry across treatments, accounting for just over 50% of the variation in preference (Figure 2). (Protein: entered first; partial R^2 = 0.281; model R^2 = 0.281; parameter estimate −3.573, F = 9,883; P = 0.006. Salicin: entered second; partial R^2 = 0.252; model R^2 = 0.533; parameter estimate −0.230; F = 9.683; P = 0.006.) It is important to note that of these two variables, only salicin concentration varied significantly with light treatment; protein concentration was not affected by light (Table 2c and d).

DISCUSSION

This study shows there are distinct differences in how different categories of plant characteristics respond to variable light environments, with potentially important implications for understanding variation in herbivore responses. The effects of light availability on plant size, growth rates, and gross leaf chemical variables were generally in accord with patterns commonly observed for sun versus shade leaves (Grime, 1966; Larcher, 1980) and the CNBH. For example, leaves from plants raised in high light had higher specific weights (i.e., thicker leaves), lower water content, higher C, lower N, higher C/N, higher TPG, and higher PG allocation than leaves from plants raised in low light. Leaves from plants switched between sun and shade were either like sun leaves, like shade leaves, or intermediate between the two for all these variables. It is striking that we could never reject the hypothesis that the effects of short- and long-term light availability were additive for all of these variables, because there were no significant overall initial × final light interactions for plant size at harvest, growth rates, or gross leaf chemistry.

In marked contrast to these above variables, concentrations of individual phenol glycosides could not be explained simply on the basis of initial and/or final light treatments. This was the only group variable that showed an overall significant initial × final light interaction effect. Each compound showed a different pattern with respect to the effects of initial, final, and interactive light effects. Of the four compounds, three (salicortin, tremuloidin, and tremulacin) exhibited patterns in accord with the CNBH, being highest in HH plants, lowest in LL plants, and intermediate in switched plants. Salicin did not conform to this pattern, because switched plants had higher concentrations than either HH or LL plants. Although the effect of light-switching on salicin concentration was quite unexpected, it is unlikely to have been an artifact because the increase occurred only in two of the three shade treatments (all of which were extracted and analyzed in an identical manner) and because the extraction method had no detectable effect on phenol glycoside concentrations and composition (see Methods and Materials). Other studies with this clone (Wait et al., 1998) have also shown that salicin, but not the other three PGs, varies in a manner that is inconsistent with the CNBH when plants are fertilized with N.

It is worth noting that the summed total phenol glycoside concentration and PG allocation (as a fraction of total C) did not show any significant initial × final light interactions. Consequently, aggregating these specific chemical variables into a grosser estimate of C-based defense compound allocation, as is often done with respect to testing the CNBH, obscured important differences in the way individual compounds changed with respect to light treatment. Nevertheless, because salicortin, tremuloidin, and tremulacin comprised the bulk of the TPGs and did conform to CNBH expectations, TPGs and PG allocation also conformed to CNBH expectations.

Patterns of beetle feeding preference could not be explained simply by using CNBH predictions based on equilibrium resource availability. Although beetles tended to prefer LL to HH leaves, as expected, albeit weakly, they strongly preferred HH to LH leaves, as opposed to either showing no preference (if LH leaves were the same as HH leaves) or preferring HH leaves (if LH were the same as LL leaves). A similar pattern of preference, albeit nonsignificant, was shown for HH over HL leaves. The specific preference for HH over LH leaves, and the overall preference patterns across the three trials, were not obviously related to either the gross leaf chemical variables of total C, total N, water, C/N ratio, TPGs or PG allocation, or to individual concentrations of salicortin, tremuloidin or tremulacin.

Differences in salicin and protein concentrations were the only variables to emerge as significant in regression analyses relating overall preference patterns with chemistry. Of these two variables, only salicin showed significant light treatment effects. Salicin was also the only chemical variable that was higher in the least preferred switched plants, being highest in HL plants, where preference for HH plants was most marked. These data suggest that high concentrations of salicin in switched plants led to preference for plants with lower salicin content. This explanation is consistent with the strong preference for HH over HL plants, where the HL plants had the highest salicin content, as well as the trend for preference for HH over LH plants. While such an explanation cannot account for the weak preference for LL over HH plants, where salicin did not differ, it is consistent with other studies that have shown that high concentrations of salicin inhibit feeding by this beetle (Tahvanainen et al., 1995; Wait et al., 1998).

Reichardt et al. (1991) reported that only one of six C-based secondary metabolites in balsam poplar was reduced after two years of shade. Snowshoe hares, which are known to be responsive to some of these compounds (Bryant, 1987; Bryant et al., 1987), showed only a slight, nonsignificant preference for shaded plants. In their study, it is clear that many of these compounds did not conform to CNBH predictions for long-term changes in light availability (shading), apparently due to small-scale fluctuations in environmental conditions (Reichardt et al., 1991). They concluded that the time scale of their experimental manipulation was too long relative to the time scale of changes in chemical allocation. In an analogous way, we suspect that, in our study, environmental changes occurred over too short a time scale for CNBH predictions to hold for all compounds. For unknown reasons, salicin had not yet equilibrated to ambient light conditions in HL and LH plants by the time of feeding preference assays, and this nonequilibrium concentration was a contributory factor in beetle preference.

Both our study and that of Reichardt et al. (1991) show that although the CNBH can predict many aspects of gross leaf chemistry, predicting specific patterns of herbivore preference can critically depend on the time scales of changes in environmental conditions relative to the time scale of acclimation of chemi-

cals to which the herbivore responds. It seems reasonable that we could enhance predictability of these relationships by knowing more about the temporal relationships between acclimation processes and resulting leaf chemistry.

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DEFENSE SECRETION OF *Prorhinotermes simplex:* TOXICITY TO INSECTICIDE SUSCEPTIBLE AND RESISTANT HOUSE FLY[1]

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Abstract—(*E*)-1-Nitropentadec-1-ene (NPE), the main component of the defense secretion of *Prorhinotermes simplex* soldiers, is toxic to both insecticide-susceptible (S) as well as to insecticide-resistant (R) strains of the house fly, *Musca domestica*. The LD_{50} is 11.7 μg/female fly for the S strain and 9.7 μg for the R strain. The same efficacy of NPE on R and S strains indicates a different mechanism of action compared to conventional chlorinated, organophosphorus, and pyrethroid insecticides. Termite nestmates are protected against NPE by a specific detoxification mechanism. 1-Nitropentadecane, the detoxification product of NPE is nearly nontoxic to house flies, and doses up to 160 μg/fly caused only very low mortality.

Key Words—Termites, Isoptera, Rhinotermitidae, *Prorhinotermes simplex*, defense secretion, (*E*)-1-nitropentadecene, toxicity, *Musca domestica*, insecticide resistance.

INTRODUCTION

The defense of a termite colony that is carried out by the soldier caste is phylogenetically an important feature enabling termites to survive, despite the fact, that they are a substantial part of the food chain for many animals, including predatory insects. Different defensive adaptations have evolved from simple biting to the production of toxic secretions. Chemical defense is typical for numerous genera of the family Rhinotermitidae (Deligne et al., 1981). The mandibulate

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soldiers of the primitive subterranean termite *Prorhinotermes simplex* (Hagen) protect the nest by biting and smearing a cephalic secretion on intruders. The secretion is produced in the frontal gland, stored in a large frontal gland reservoir, and released from a pore situated dorsally on the head. More than 90% of the secretion is a single nitroalkene, (*E*)-1-nitropentadec-1-ene (NPE) (Vrkoč and Ubik, 1974). Contact toxicity of the secretion to the house fly was reported from preliminary tests by Hrdý et al. (1977). The secretion is not toxic to termites of the same species. The ability of nestmates to survive in the presence of toxic nitroalkenes and the respective detoxifying enzymes were studied in two species of the family Rhinotermitidae by Spanton and Prestwich (1982), who found specific oxidoreductases capable of reducing the carbon–carbon double bond of NPE, forming nontoxic 1-nitropentadecane (NPA).

The specific effects of NPE, which are different from those of conventional insecticides, and the safety of its detoxification product NPA were tested on insecticide-susceptible and resistant strains of *Musca domestica* L.

METHODS AND MATERIALS

Compounds. (*E*)-1-Nitropentadec-1-ene (NPE) and 1-nitropentadecane (NPA) were prepared according to published procedures (Spanton and Prestwich, 1982). For preparation of NPE, tetradecanal (2 g, 9.4 mmol) was dissolved in methanol (10 ml), then nitromethane (25 ml) and a methanolic solution of potassium hydroxide (3 N, 5 ml) was added. After 1.5 hr of reaction at room temperature, the suspension was acidified (pH 2), extracted with ether (3 × 50 ml), and extracts were dried over magnesium sulfate. Chromatographic purification on a silica gel column, and elution with petroleum ether–ether mixtures (0–70% of ether) yielded 1-nitro-2-hydroxypentadecane (2.003 g, 70%), accompanied by NPE (0.329 g, 15%). 1-Nitro-2-hydroxypentadecane (300 mg, 1.1 mmol) was heated in a mixture of pyridine (5 ml)–acetanhydride (5 ml) at 60°C for 0.5 hr, and the reaction mixture was cooled to room temperature, acidified with hydrochloric acid (36%), and extracted with hexane (3 × 50 ml). NPE (123 mg, 44%) was obtained after chromatography on a silica gel column and elution with petroleum ether–ether mixtures (0–30% ether).

NPA was prepared by the following reactions. NPE (208 mg, 0.81 mmol) was reduced with sodium borohydride (71.5 mg, 1.89 mmol) in a mixture of tetrahydrofuran–methanol (9 : 1, 4 ml) for 1 hr at room temperature and the reaction was quenched with water (0.5 ml). This provided a crude product (170 mg), which was purified by chromatography on a silica gel column, and eluted with petroleum ether–ether mixtures (0–20% ether). The yield of pure NPA was 105 mg (50% yield).

The purity of prepared compounds (>99%) and stereochemistry were checked by nuclear magnetic resonance spectrography and gas–liquid chromatography.

Insects. Prorhinotermes simplex (Hagen) colonies were collected in 1964 from decayed pine wood in a forest near Soroa, Pinar del Rio Province, Cuba, and kept in laboratory colonies. The frontal gland secretion was isolated from the termites (Vrkoč and Ubik, 1974).

House Fly, Musca domestica. The insecticide-susceptible house fly strain (S) of Pavia SRS/WHO and the resistant (R) strain Okrouhlo, derived from a field population, were raised in the laboratory of the National Institute of Public Health, Praha (Rupeš et al., 1983). The R strain was characterized by resistance factors (RF), calculated according to the LD_{50} for several insecticides, including the chlorinated hydrocarbon lindane (RF = 7); the organophosphorus compounds fenitrothion (RF = 54) and dimethoate (RF = 20), and the pyrethroids cypermethrin (RF = 253), deltamethrin (RF = 15), and tetramethrin (RF = 58).

Toxicological Tests. Doses of 2.5–40 μg NPE and 10–160 μg NPA were administered topically in 1 μl of acetone to female flies that were 1–4 days old. Between 54 and 126 flies were treated per dose. Mortality was scored after 48 hr, and the response was expressed as a calculated regression line. The data were corrected for control mortality according to Abbott's formula and processed by probit/log dose transformation (Busvine, 1957).

RESULTS AND DISCUSSION

The toxicity of NPE, the main component of soldier frontal gland secretion, to *M. domestica* was confirmed (Table 1), but the efficacy of NPE is considerably lower than that of conventional insecticides. The LD_{50} of 11.7 μg/female fly for S-strain flies is approximately four to five orders higher than the lethal dose for dimethoate (LD_{50} = 0.00026 μg), fenitrothion (LD_{50} = 0.00208 μg) or cypermethrin (LD_{50} = 0.00154 μg). However, toxicity of NPE to both S and R strains of *M. domestica* is approximately the same (LD_{50} = 9.7 μg and 11.7 μg, respectively). The difference in average mortality between S and R strains is not significant (tested by analysis of variance when the same doses were used, $P = 0.288$). The slopes of the regression lines are steep, showing apparent toxic effects dependent on increasing doses (Figure 1).

The similar efficacy of NPE on S and R strains indicates a different mode of action of this termite nitroalkene compared to conventional chlorinated, organophosphate, and pyrethroid insecticides attacking the nervous system. Moreover, intoxication by these nerve poisons is characterized by excitation, disturbance of coordinated movement, and knock down (Büchel, 1983), while flies intoxicated by NPE are quiet and die without visible excitation. According to Spanton and Prestwich (1982), the lipophilic NPE possesses a reactive electrophilic center that is responsible for causing deterioration of physiological function that can lead to death in the target insect.

TABLE 1. TOXICITY OF (*E*)-1-NITROPENTADEC-1-ENE (NPE) AND 1-NITROPENTADECANE (NPA) TO *Musca domestica* FEMALES OF INSECTICIDE-SUSCEPTIBLE (S) AND RESISTANT (R) STRAINS

Compound	*M. domestica* strain	Equation of probit regression line[a]	LD_{50} (μg/insect)	95% confidence interval	χ^2 (degrees of freedom)	Hypothesis of linearity
NPE	S	$y = 0.778 + 3.958 \log D$	11.7	9.6–14.2	χ^2 (5) = 16.5[c]	rejected
NPE	R	$y = 2.287 + 2.747 \log D$	9.7	8.7–10.8	χ^2 (5) = 7.3	accepted
NPA	S	$y = 2.498 + 0.508 \log D$	(83515.8)[b]		χ^2 (3) = 3.1	accepted
NPA	R	$y = 1.932 + 1.046 \log D$	(854.8)[b]		χ^2 (3) = 1.7	accepted

[a] y, mortality in probits corrected according to Abbott's formula. D, dose/μg.
[b] LD_{50} was not possible to assess experimentally.
[c] The confidence interval calculated with regard to the χ^2 test.

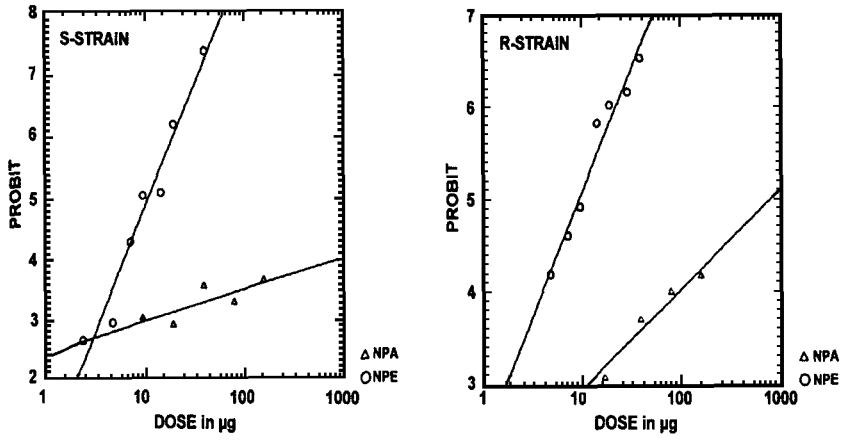

FIG. 1. Toxicity of (*E*)-1-nitropentadec-1-ene (NPE) and 1-nitropentadecane (NPA) to insecticide-susceptible (S) and resistant (R) strains of *Musca domestica*. Mortality in probits.

NPA, the detoxification product of the defensive secretion, is practically nontoxic to flies of both strains. Doses up to 160 µg/female caused only low mortality, with 8.6% in the S strain and 20.6% in the R strain. LD_{50} values are shown in Table 1. Differences in average mortality between strains are not significant ($P = 0.765$). The action of NPA on both strains is 100- to 1000-fold lower than that of NPE. This confirms the report of Spanton and Prestwich (1982) on the 10- to 100-fold difference in action of defensive secretions to biochemically protected and unprotected insects. The calculated hypothetical LD_{50} for house flies is 0.05–5 g NPA/g insect. It is apparent that the detoxification mechanism in *Prorhinotermes simplex* is efficient and protects nestmates in the colony against the nitroalkene secretion produced by their own soldiers.

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INCUBATED HUMAN SWEAT BUT NOT FRESH SWEAT ATTRACTS THE MALARIA MOSQUITO *Anopheles gambiae* SENSU STRICTO

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Abstract—A dual-port olfactometer was used to quantify behavioral responses of the malaria mosquito *Anopheles gambiae* sensu stricto to volatiles emitted by sweat samples collected from three human volunteers. Significant attraction ($P < 0.05$) was found to sweat of two volunteers after two days and to one of the volunteers' sweat after one day of incubation at 37°C. There was no attraction to any of the fresh sweat samples. The pH of the sweat that became attractive after one or two days had changed from acidic (5.5–5.75) to alkaline (8.25–8.75), while the pH of the sweat of the third volunteer (pH 7) did not change during incubation. Microorganisms, present in all fresh sweat samples, showed distinct growth during incubation. The concentration of lactic acid decreased an average of 23% in two days of incubation. The role of the pH, skin microflora, and lactic acid in the differential attractiveness of human sweat samples is discussed.

Key Words—*Anopheles gambiae* s.s., malaria mosquito, host-seeking behavior, semiochemicals, humans sweat, skin microflora, lactic acid, pH.

INTRODUCTION

Females of the malaria mosquito *Anopheles gambiae* Giles sensu stricto seek humans during the night and rely mainly on olfactory cues, so-called kairomones, to find their hosts (Takken, 1991; Knols, 1996). Despite intensive research, the identity of these kairomones is not known. Carbon dioxide present in mammalian breath has been shown to be an attractant for many mosquito species. It appears

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to be of minor importance, however, for the anthropophilic *An. gambiae* s.s. (Mboera and Takken, 1997). It is widely assumed that important kairomones for *An. gambiae* s.s. emanate from the human skin (Knols, 1996). In addition to carbon dioxide, only lactic acid and 1-octen-3-ol have repeatedly been reported as kairomones for mosquito species other than *An. gambiae* s.s. (Acree et al., 1968; Kline et al., 1990; Geier et al., 1996).

The yellow-fever mosquito *Aedes aegypti* (Linnaeus) is attracted to human sweat samples (Skinner et al., 1965; Müller, 1968; Eiras and Jepson, 1991, 1994), although large variations in responses were reported. Sweat has long been suspected as a kairomone source for *An. gambiae* s.s., but evidence was not present until Braks et al. (1997) found attraction to sweat samples, collected from volunteers from Burkina Faso (Cork and Park, 1996), using a two-choice olfactometer. Their attempt to repeat the experiment with freshly collected sweat samples from other volunteers failed; no attraction was found (M. A. H. Braks, unpublished data). The latter sweat samples were acidic (pH 5.5), which is in accordance with other studies (Noble and Somerville, 1974), but the sweat sample from Burkina Faso had an alkaline pH of 8. To our knowledge, alkaline sweat is only observed as a result of microbial decomposition of sweat components into ammonia (Bergeim and Cornbleet, 1943; Weiner and Hellmann, 1960; Müller, 1968).

An. gambiae s.s. has also been shown to be attracted to the volatiles of Limburger cheese, which, to a human, are reminiscent of human foot odor (De Jong and Knols, 1995), and to a synthetic blend of fatty acids occurring both in this cheese and in human foot odor (Knols et al., 1997). These fatty acids are produced during ripening of the cheese by the inoculated bacteria (Cogan and Daly, 1987). Some fatty acids in foot odor have also been shown to be of bacterial origin (Noble and Somerville, 1974). Braks et al. (1997) showed that in addition to fatty acids, however, other chemicals must play a role in odor-guided host-seeking of malaria mosquitoes because sweat samples kept their attractiveness even when fatty acids in the headspace were artificially minimised (Braks et al., 1997).

The present study of the response of *An. gambiae* s.s. to sweat determined the identity, origin, and biosynthesis of the kairomones. The effect of incubation of sweat samples on their attractiveness for *An. gambiae* s.s. was investigated, together with the variation between samples from three different human individuals. The role of pH, lactic acid concentration, and bacterial growth in the differential attractiveness of sweat samples is discussed.

METHODS AND MATERIALS

Mosquitoes. The *An. gambiae* s.s. strain we used originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi, Rome) and was maintained at 27 ± 1°C,

80 ± 5% relative humidity, and a photo/scotophase of 12 L : 12 D. Adults were kept in 30-cm^3 gauze-covered cages and had access to 6% (v/v) glucose solution. Females were offered blood from a human arm twice a week for 10 min. Eggs were laid on wet filter paper, hatched in trays of water, and the larvae were fed Tetramin fish food. Pupae were collected daily from the trays and were allowed to emerge in the adult cages. In the bioassay the 4- to 8-day-old female mosquitoes had not received a bloodmeal. One day before the experiments, females were randomly selected from the adult cages in batches of 30 and put in small release containers. They had access to cotton wool soaked with tap water only.

Olfactometer. A dual-port olfactometer (Figure 1), modified from Knols et al. (1994), was used to study the attractiveness of sweat samples. The olfactometer consisted of a flight chamber (1.6 × 0.6 × 0.6 m) in which mosquitoes were released. Air, obtained from a pressurized air source, reached room temperature when passing through 10-m-long silicon tubes (7 mm diameter) in the room. Subsequently, the air was purified by means of activated charcoal and humidified by passing it through two bottles filled with demineralized water. This conditioned air (60 ± 5% relative humidity; 27 ± 0.5°C) entered the flight chamber (55 ± 5% relative humidity; 27 ± 0.5°C) through two small ports (5 cm diameter, horizontally aligned, 30 cm apart). The ports of the flight chamber were linked to trapping devices (for details see Knols et al., 1994). These were made of glass containers through which the conditioned air entered the flight chamber. The odor source, applied on the rough surface of a sand-blasted glass slide (5 × 2 cm), was placed in the glass container and odorous air was passed on to the flight chamber. Airflow was 20 cm/sec. Light (6.3 ± 1.4 lux) was produced by nine incandescent light bulbs suspended over the roof of the tunnel.

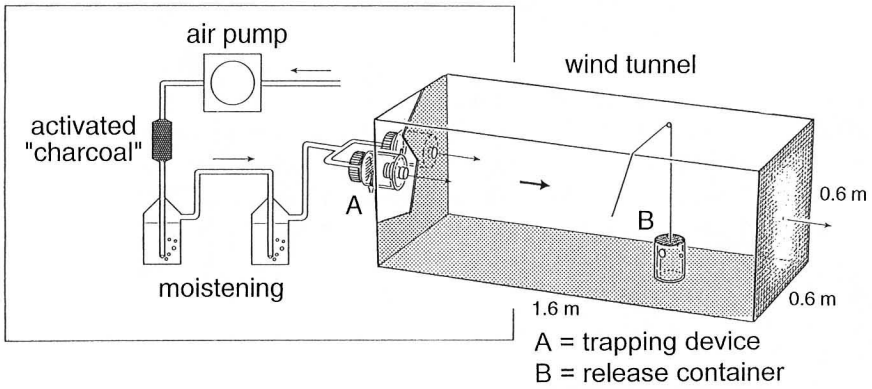


FIG. 1. Diagram of the dual-port olfactometer.

In each test a batch of 30 mosquitoes was released from a container placed in the flight chamber, 1.0 m downwind from the two ports. The trap catches were counted, and the distribution between the test and control stimuli was determined. The mosquitoes that had not entered one of the two ports were removed from the flight chamber after each test. Each subsequent test started with new mosquitoes, clean traps, and newly applied odor stimuli.

Odor Stimuli. Sweat droplets were collected from the foreheads of three Caucasian human volunteers, one female (MB, 27 years old) and two males (OB, 25 years old, and TD, 28 years old) with sterile glass Pasteur pipets and put in glass vials (2 ml). From each volunteer 1.5 ml sweat was collected twice on different days. Sweat production was stimulated by physical exercise in a warm humid room (30°C, 90% relative humidity). Immediately after collection, the sweat sample (1.5 ml) was divided in three subsamples of 0.5 ml each. One subsample was directly stored at −5°C. The other subsamples were incubated under aerobic conditions at 37°C for one or two day(s), after which they were also stored at −5°C. Afterwards, the pH (Indicator Paper, Merck) and lactic acid concentration (Lactic Diagnostic Kit Sigma, NR 735) of each sample was determined.

The presence and growth of microorganisms was indicated by the number of colonies on agar plates (Iso Sensi Test, Biotrading) that were inoculated with 50 μl of 10%, 1%, and 0.1% diluted sweat samples. Control plates were inoculated with 50 μl of sterile distilled water. The agar plates were incubated for one day at 37°C under aerobic conditions.

Experimental Procedures. For testing attractiveness of sweat, 0.1 ml of sweat (fresh or incubated for one or two days) was pipetted onto the glass slide and tested directly against the control, which consisted of an equivalent amount of distilled water. Control tests were also performed to assess the response of the mosquitoes to clean air over untreated slides or to distilled water alone. Glass slides were placed inside the glass traps through which air was led into the flight chamber.

Behavioral responses of mosquitoes were tested during the last 4 hr of the scotophase. Each experiment consisted of five 15-min tests examining the three differently treated sweat subsamples, which originated from one collection day of one volunteer, and the two control trials. From each collection from each volunteer, this set of five tests was replicated on four days. The odor stimuli were tested the same number of times from the right and the left port to rule out positional effects, and the order of the different tests was randomized per experimental day.

Statistical Analysis. For each 15-min test, the numbers of mosquitoes caught in the test and control port were recorded as proportions of the total number of mosquitoes caught. Subsequently, an arcsine square root transformation for normalization was applied on the proportions. A Student's t test was

performed to examine differences between test and control odors. The sweat samples were collected from three different volunteers, and the data were analyzed separately for each volunteer (Sokal and Rohlf, 1995).

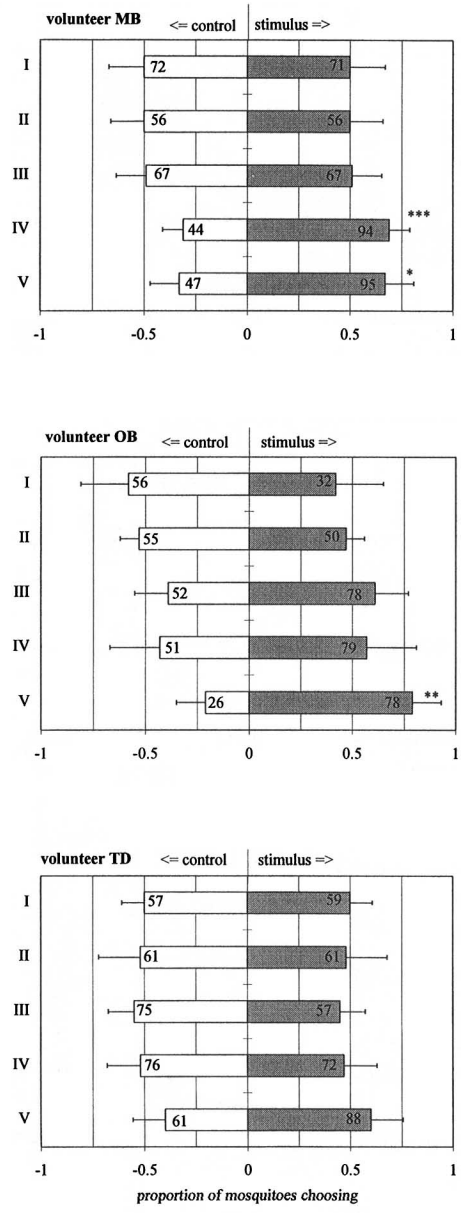
RESULTS

Bioassay. No significant differences were found between the fresh sweat samples and the control for any of the volunteers (Figure 2). For volunteer MB, the sweat samples that had been incubated for one and two days were significantly more attractive than the control ($P < 0.05$). For volunteer OB, a significant attraction was found for the sweat samples that had been incubated for two days ($P < 0.01$), but not for those after one day of incubation. For volunteer TD, none of the sweat stimuli tested were attractive. The control tests showed that *An. gambiae* entered the trap in response to clean or slightly humidified air currents, but that there were no differences between the ports.

Measurements. Table 1 shows the pH and lactic acid concentration of the different sweat samples and the numbers of skin microorganisms present in fresh sweat samples. The pH of the sweat samples of volunteers MB and OB showed a shift from acidic (pH 5.5–5.75) when fresh to alkaline (7.75–8.75) after one or two day(s) of incubation. The pH of the two fresh sweat collections of TD were not acidic, but neutral, and the pH did not change to alkaline during incubation. The fresh sweat samples of all volunteers contained substantial amounts of lactic acid (1.59–3.38 mg/ml) which decreased on average to 77.2 ± 12.5% in two days of incubation. Microorganisms were abundant in all fresh sweat samples, and no colonies were found on the control plates. The numbers of skin microorganisms present in sweat samples incubated for one or two day(s) could not be determined as the agar plates of all dilutions were completely overgrown, indicating that exponential growth of microorganisms had occurred.

DISCUSSION

Surprisingly, there was no attraction to any of the fresh sweat samples of the different volunteers, which seems to contradict earlier reports on behavioral responses of *An. gambiae* s.s. to human sweat (Braks et al., 1997). However, the results show that sweat can become attractive after incubation at body temperature for one or two day(s). We suggest that the sweat sample used by Braks et al. (1997) may have undergone alterations between collection and testing, which resemble the incubation process. This conclusion is supported by the fact that the latter sweat sample had an alkaline pH of 8, like our incubated sweat, instead of a normal slightly acidic pH (Noble and Somerville, 1974). Bergeim and Cornbleet (1943) showed that incubated sweat becomes alkaline due to ammonia formation



LEGEND FIG. .2.:

trial	control	versus	stimulus
I	clean air	versus	clean air
II	water	versus	water
III	water	versus	fresh sweat
IV	water	versus	1 day old sweat
V	water	versus	2 day old sweat

by bacteria from the urea present. In addition, they found that the lactic acid concentration of sweat decreased during incubation, due to bacterial action. The acidity of freshly secreted sweat is due to the production and secretion of lactic acid by the eccrine sweat glands (Thurmon and Ottenstein, 1952). All our sweat samples showed a decrease in lactic acid concentration after two days of incubation. Müller (1968) also reported a pH shift from acidic to alkaline in incubated sweat samples. However, he found that *Ae. aegypti* was attracted to freshly collected sweat from the fore arm and that the attractiveness was lost after incubation. This loss in attractiveness was thought to be due to the absence of lactic acid and other acidic components in the headspace of alkaline sweat (Müller, 1968; Thompson and Brown, 1955). Lactic acid has repeatedly been shown to be an important kairomone for *Ae. aegypti* (Acree et al., 1968; Müller, 1968; Geier et al., 1996). Since we only found attraction to alkaline sweat and because the artificial alkalinization to pH 10 did not affect the attractiveness of human sweat (Braks et al., 1997), we doubt that lactic acid is very important in the attraction of *An. gambiae* s.s.

Although attraction of *An. gambiae* s.s., has been observed to alkaline sweat samples only, not all alkaline sweat samples were attractive. Most likely other chemical changes than the observed shift in pH occur during incubation and the resulting increased bacterial activity. Nevertheless, growth of microflora has also been observed in unattractive incubated sweat samples of one volunteer (TD), while there was no change in pH. The results suggest that the presence and growth of skin flora, pH, and individual differences in sweat composition together give rise to differential responses of *An. gambiae* s.s. to sweat samples of one volunteer and between volunteers. Our findings clarify, at least partly, the differences found between previous studies (Skinner et al., 1965; Müller, 1968; Eiras and Jepson, 1991, 1994; Braks et al., 1997) as conclusions are usually based on responses to sweat of a single volunteer. In addition, confusion is raised by studies reporting responses to sweat derived from skin emanations collected on adsorbent material such as filter paper, cotton wool, glass beads, or Tenax (Thompson and Brown, 1955; Parker, 1948; Roessler, 1961; Skinner et al., 1965; Bar-Zeev et al., 1977; Schreck et al., 1981; Geier et al., 1996), while pure sweat droplets have not been tested.

FIG. 2. Attractiveness of *An. gambiae* s.s. to clean air, water, and the sweat samples of three human volunteers, expressed as the mean proportion of mosquitoes that was caught in the stimulus port (positive proportion) and control (negative proportion) relative to the total number that had entered the olfactometer ports in the eight replicates. The horizontal lines represent the standard errors. The numbers placed in the bars indicate that total number of mosquitoes caught of 240 (8×30) mosquitoes released. Asterisks mark significant differences found between the test and control stimulus (Student's t test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

TABLE 1. VALUES OF pH AND LACTIC ACID CONCENTRATION (mg/ml) OF FRESH AND ONE OR TWO DAY(S) INCUBATED (37°C) SWEAT SAMPLES OF THREE VOLUNTEERS (MB, OB AND TD), COLLECTED ON TWO DIFFERENT DAYS (1 AND 2).[a]

	Volunteers					
	MB		OB		TD	
Collection	1	2	1	2	1	2
pH						
fresh sweat	5.5	5.5	5.75	5.75	7.0	7.0
1 day incub. sweat	8.25	8.5	7.75	8.25	7.0	7.25
2 days incub. sweat	8.75	8.75	8.25	8.75	7.5	7.5
Lactic acid	mg/ml (%)[b]	mg/ml (%)	mg/ml (%)	mg/ml (%)	mg/ml (%)	mg/ml (%)
fresh sweat	2.00	2.07	2.37	2.82	2.74	3.83
1 day incub. sweat	1.83 (91.5)	1.75 (84.5)	1.94 (81.8)	3.17 (112.0)	2.37 (86.5)	3.58 (93.5)
2 days incub. sweat	1.59 (79.5)	1.77 (85.5)	1.73 (73.0)	2.44 (86.5)	1.48 (54.0)	3.25 (84.9)
Microorganisms[c]						
fresh sweat	1.7×10^4	0.9×10^4	3.0×10^4	1.0×10^4	1.6×10^7	1.7×10^4

[a]The number of skin microorganisms in fresh sweat samples are indicated in the last line.
[b]% = (concentration lactic acid in incubated sweat sample/concentration lactic acid in fresh sweat sample) × 100%.
[c]Colony forming units/ml.

The typical odor of the armpit is the product of bacterial decomposition of odorless apocrine sweat (Stoddart, 1990), but little is known about the role of the skin flora in the production of other human odors from eccrine sweat and sebum. Kahn et al. (1969) suggested that increased bacterial decomposition of sweat may cause an increased attraction of mosquitoes to a human arm while sweating, but conclusive evidence is lacking. The present study provides strong indications that the skin flora plays a role in the production of volatiles as fresh sweat is not attractive in any case. We hypothesize that a continuous bacterial action on secretions on the human skin results in volatiles that function as kairomones for mosquitoes. Future research is underway to test this hypothesis.

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SIGNAL TRANSDUCTION OF BARNACLE EGG-HATCHING PHEROMONE: PHARMACOLOGICAL ASSAYS INDICATE A COMPARATIVELY SIMPLE MECHANISM OF EICOSANOID ACTION

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Abstract—Eicosanoids are generally regarded as autocoids that act via G-protein-linked receptors. There are exceptions to this model, however, both in terms of function and mechanism of action. The present paper concerns one such example, an hydroxy fatty acid that acts as a pheromone, not an autocoid, in inducing barnacle egg hatching. Preliminary findings of pharmacological assays on the mechanism of action of this eicosanoid pheromone do not support the involvement of G proteins, Ca^{2+}, or protein kinase C in signal transduction. Instead it is concluded, tentatively, that barnacle egg hatching pheromone acts via a comparatively simple pathway involving the second messenger, cyclic AMP.

Key Words—Eicosanoid, pheromone, egg hatching, barnacle, signal transduction, G protein.

INTRODUCTION

Eicosanoids—C_{20} polyunsaturated fatty acids—are important molecular mediators of diverse physiological and pathological processes (for reviews see Smith, 1989; Negishi et al., 1993; Piomelli, 1993; Stanley-Samuelson, 1994). The overwhelming majority of research done on eicosanoids relates to mammals. Here, generally speaking, eicosanoids act as local hormones—autocoids—and act through G-protein-linked receptors (Smith, 1989; Shimizu and Wolfe, 1990). Studies on lower vertebrate and invertebrate species, particularly aquatic forms, have highlighted additional roles (for reviews see Bundy, 1985; Ruggeri and

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Thoroughgood, 1985; Stanley-Samuelson, 1987, 1991, 1994; Stanley-Samuelson and Loher, 1990). For example, eicosanoids may function as second messengers (Piomelli et al., 1987a,b) and as pheromones in barnacles (e.g., Clare et al., 1982; Clare, 1997) and fish (e.g., Sorensen, 1992, Kitamura et al., 1994; Ogata et al., 1994; Moore and Waring, 1996). Investigations on the biochemical mechanism of action of eicosanoids in nonmammalian species indicate that receptors and transduction pathways may be comparable to those of mammals (see e.g., Stanley-Samuelson and Pedibhotla, 1996; Qian et al., 1997), but this statement is based on limited information. Even among mammals, there are apparent exceptions to the model of eicosanoid action. Hydroxy fatty acids, for example, have been reported to act independently of G-protein-linked receptors, perhaps by altering the physical properties of membranes (Spector et al., 1988, but see O'Flaherty and Rossi, 1993). Furthermore, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Forman et al., 1995; Kliewer et al., 1995) and leukotriene B_4 (Devchand et al., 1996) act via nuclear receptors, while PGA_2 interacts with nuclear DNA (Holbrook et al., 1992).

In view of the above deviations from the model of eicosanoids and their action (Smith, 1989), it seemed of interest to examine the mechanism of action of an eicosanoid pheromone—barnacle egg-hatching pheromone (EHP). The EHPs of a number of species have been examined and identified as hydroxy fatty acids (e.g., Song et al., 1990), although the precise identity of any EHP has yet to be determined (Gerhart et al., 1990; Clare, 1995, 1997). EHP is thought to be synthesized from endogenous eicosapentaenoic acid (Clare and Walker, 1986) that has been released from membrane phospholipid (Clare, 1997). Alternatively, bearing in mind the ability of hydroxy fatty acids to be taken up by membrane lipid (Spector et al., 1988), EHP could be released directly from membrane stores. Whatever the origin of EHP, the stimulus to release is presumed to be related to feeding (Crisp and Spencer, 1958; Clare, 1995, 1997). EHP provides a remarkable mechanism for synchronizing egg hatching with the spring phytoplankton bloom in the boreoarctic barnacle, *Semibalanus balanoides* (Crisp and Spencer, 1958; Clare, 1997). A comparable role in other barnacle species has yet to be examined critically. The present study aimed at a preliminary characterization of the biochemical mechanism of action of barnacle EHP. In particular, putative components of the signal transduction pathway were examined in vivo by pharmacological assays.

METHODS AND MATERIALS

Animals. Gravid *Semibalanus balanoides* used in this study were either collected from the Menai Strait, North Wales, or were supplied, attached to *Mytilus edulis* shells, by Millport Marine Station. They were maintained at the MBA at

9°C until used for bioassay. *Elminius modestus* were obtained, as required, from the carapaces of *Carcinus maenas* in the holding tanks of the MBA.

Test Compounds. All compounds, except the following, were obtained from Sigma: forskolin (ICN), A23187 (free acid), thapsigargin, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), and phorbol-12-myristate-13-acetate (PMA) (Calbiochem). Test solutions were prepared from aqueous stocks except for the following: gossypol, OAG, PMA, thapsigargin, verapamil, and nifedipine (ethanol), A23187, and forskolin (DMSO). Aluminium fluoride was prepared by mixing 10 mM aluminium chloride with 10 mM sodium fluoride (Clare, 1998). Calcium-free seawater (not buffered with EGTA) was prepared according to Clare (1996).

Egg-Hatching Pheromone Extraction. Crude, or partially purified, EHP was used in the assays. Crude EHP was prepared by grinding four whole, adult barnacles in 2 ml of 0.45-μm-filtered Eddystone seawater (FESW; see Clare, 1996, 1997) with a mortar and pestle. The extract was left at ~20°C for 15 min and then centrifuged at 16,000*g* for 5 min. Starting material for partially purified *S. balanoides* EHP extraction (see Clare, 1997) was 500 g (wet weight) of whole adults. The final ethyl acetate phase was rotary evaporated to 10 ml and stored at −20°C. *Elminius modestus* EHP was extracted from 125 g (wet weight) of whole adults using a different protocol. After extracting 3× with FESW (1 × 100 ml and 2 × 50 ml), the extracts were combined and filtered through Whatman No. 3 filter paper. The combined extract was then centrifuged at 4000*g* for 5 min and an equal volume of acetone was added to the supernatant. The resultant precipitate was removed by centrifugation (4000*g*, 5 min), and the yellow supernatant was filtered through Whatman No. 1 filter paper. The acetone was removed by rotary evaporation at 60°C, and the aqueous extract was acidified to pH 3.9 with glacial acetic acid. After adjusting to 15% ethanol, eicosanoids were extracted using a Sep-Pak C_{18} protocol (Powell, 1987). Four 50-ml samples were applied to the cartridge yielding 40 ml ethyl acetate extract. The cartridge was reconditioned between samples (Powell, 1987). This extract was reduced to 2 ml by rotary evaporation and then stored at −20°C.

Egg-Hatching Bioassays. Most of the above compounds were first assayed for their ability to induce egg hatching (see Table 1 below). The assays were carried out as described previously and were repeated at least once with egg masses from different barnacles (Clare et al., 1985; Clare, 1997). Fully mature egg masses of *S. balanoides* and *E. modestus* were removed and washed in running seawater for at least 1 hr prior to bioassay to remove endogenous EHP. Immature egg masses of *E. modestus* were cultured to maturity in 10-ml-capacity glass scintillation vials containing 5 ml FESW. For assays that incorporated a negative control in the experimental design, i.e., an egg mass in seawater, or seawater containing carrier solvent (at <5 ppt) if one was used to dissolve the test compound, the egg mass was subsequently tested for viability with conspecific EHP (see Clare, 1997). Crude EHP was used undiluted. For assays that

used partially purified EHP, 20 μl of ethyl acetate extract [≡ 1 g (wet weight) *S. balanoides* and 1.25 g (wet weight) *E. modestus*] were applied to a watch glass and evaporated to dryness under nitrogen (oxygen free). The oily residue was resuspended in FESW or test solution for assay (see below).

Several compounds were assayed for their ability to modulate EHP-induced egg hatching (see Table 1 below), and these assays require further elaboration.

GTP and GDP Analog. 5-Guanylylimidodiphosphate (Gpp[NH]p) was assayed at 5×10^{-5} M for its effect on egg hatching elicited by a serial dilution of EHP (*S. balanoides* = 1/8 – 1/128 crude EHP; *E. modestus* = 1/4 – 1/128 partially purified EHP). Guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) was assayed on *E. modestus* at three concentrations (10^{-5}, 10^{-4}, and 10^{-3} M) for its effect on EHP-induced (1/16 dilution partially purified EHP) egg hatching. Guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S) was assayed at 2×10^{-4} M, after preincubation of eggs for 1 hr, to determine whether it would inhibit EHP-induced (1/16 dilution partially purified EHP) egg hatching. Control assays, in all instances, employed EHP alone.

Calcium Channel Blockers. Eggs of *S. balanoides* were preincubated for 10 min with cobalt chloride and lanthanum chloride (Table 1 below). They were then assayed for egg hatching with partially purified EHP in the presence of channel blocker. Verapamil and nifedipine were assayed without addition of EHP because these organic calcium channel blockers were stimulatory in other invertebrate systems where calcium channel blockers were expected to be inhibitory in their action. (Ilan et al., 1993; Clare, 1996).

Gossypol. This PKC inhibitor (Ibrahim and Platt, 1991) was assayed on *S. balanoides* egg masses (Table 1 below). After preincubation with the drug for 5 min, an egg mass was transferred to fresh gossypol containing partially purified EHP and assayed against an EHP control.

RESULTS

The combined results of assays that aimed to examine various potential components of signal transduction of EHP are presented in Table 1. All compounds, other than those that are known to affect cyclic AMP (cAMP) levels [forskolin, isobutylmethylxanthine (IBMX), caffeine, and theophylline], were negative in the bioassays. However, dibutyryl cAMP (db-cAMP), a lipid-permeable analog of cAMP, was without effect. The GTP analog, Gpp[NH]p, did not enhance the effect of EHP on egg hatching of *S. balanoides* (Figure 1). A comparable result was obtained with *E. modestus* (data not shown). Likewise, another GTP analog, GTP-γ-S, did not potentiate EHP-induced egg hatching in the concentration range 10^{-5}–10^{-3} M (Figure 2). In accord with these results, the GDP analog, GDP-β-S did not inhibit the inductive effect of EHP on egg hatching.

TABLE 1. EFFECTS OF PHARMACOLOGICAL COMPOUNDS ON BARNACLE EGG HATCHING[a]

Compound	Species[b]	Concentration (M)	Effect	Pharmacology	Reference
AlF_4^-	S	see text	negative	locks G proteins in activated state	Loirand et al. (1990)
GTP	E	10^{-5}–10^{-3}	negative	activates G proteins	Markby et al. (1993)
Gpp[NH]p	S&E	5×10^{-5}	did not potentiate EHP-induced egg hatch	hydrolysis-resistant GTP analog	Baxter and Morse (1987)
GTP-γ-S	E	10^{-5}–10^{-3}	did not potentiate EHP-induced egg hatch	hydrolysis-resistant GTP analog	Sugimoto et al. (1993)
GDP-β-S	S&E	2.1×10^{-4}	did not inhibit EHP-induced egg hatching	inhibits, competitively, the activation of G proteins by GTP	Eckstein et al. (1979)
K^+	S	2×10^{-2}	negative	depolarizes cells	
Ca^{2+}-free ASW	S		inhibited EHP-induced egg hatch		Clare (1996)
A23187	S	0.95×10^{-6}-0.19×10^{-4}	negative	calcium ionophore	Ilan et al. (1993)
Thapsigargin	S	1.54×10^{-6} & 1.54×10^{-5}	negative	IP_3-independent releaser of $[Ca^{2+}]_i$	Takemura et al. (1989)
Cobalt chloride	S	2×10^{-3} & 5×10^{-3}	did not inhibit EHP-induced egg hatch	blocks Ca^{2+} channels	Demaurex et al. (1992)
Lanthanum chloride	S	2×10^{-3}	altered kinetics but did not inhibit EHP-induced egg hatch	blocks Ca^{2+} channels	Demaurex et al. (1992)

TABLE 1. CONTINUED

Compound	Species[b]	Concentration (M)	Effect	Pharmacology	Reference
Verapamil	S	10^{-6}–10^{-5}	negative	blocks Ca^{2+} channels	Henry (1980)
Nifedipine	S	10^{-6}–10^{-4}	negative	blocks Ca^{2+} channels	Henry (1980)
OAG	S	10^{-4} & 10^{-5}	negative	synthetic diacylglycerol; activates PKC	Kaibuchi et al. (1983)
PMA	S	10^{-4} & 10^{-5}	negative	activates PKC	Tepper et al. (1995)
Gossypol	S	10^{-4} & 10^{-5}	did not prevent EHP-induced egg hatch	inhibits PKC	Ibrahim and Platt (1991)
Forskolin	S&E	10^{-5}–10^{-3} (S); 1.2×10^{-5}–1.2×10^{-4} (E)	positive (S); $\leq 1.2 \times 10^{-5}$ negative (E)	activates adenylate cyclase	Seamon et al. (1981)
IBMX	S&E	10^{-5}–10^{-3} (S); 10^{-3} (E)	positive (10^{-5} negative) (S); positive (E)	phosphodiesterase inhibitor	Whim and Evans (1991)
Theophylline	S&E	2.78×10^{-3}	positive	phosphodiesterase inhibitor	Morse et al. (1980)
Caffeine	S&E	2.57×10^{3} (S); 3.2×10^{-4}–2.57×10^{-3} (E)	positive (S); 2.57 & 1.93×10^{-3} positive, $\leq 9.6 \times 10^{-4}$ negative (E)	phosphodiesterase inhibitor	Mathews and van Holde (1990)
db-cAMP	E	10^{-4}	negative	cAMP analogue	Yusta et al. (1988)

[a]See text for assay details.
[b]S = *Semibalanus balanoides*; E = *Elminius modestus*.

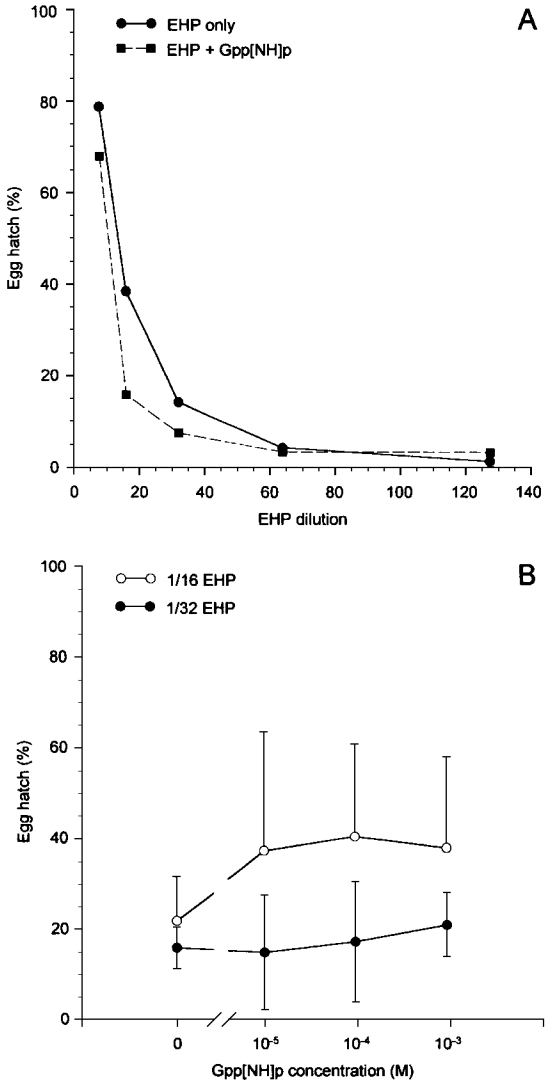

FIG. 1. Effect of GTP analogs on EHP-induced egg hatching. (A) *Semibalanus balanoides:* Plots of egg hatching induced by a dilution series of crude EHP with and without the addition of Gpp[NH]p. (B) *Elminius modestus:* Effect of Gpp[NH]p on egg hatching induced by partially purified EHP at two dilutions. Results represent means ± SEM (N = 3, except for 10^{-5} and 10^{-4} M, where N = 2). None of the Gpp[NH]p concentrations in (B) were significantly different from control values as analyzed by Dunnett's multiple comparisons test (Dunnett, 1955, 1964).

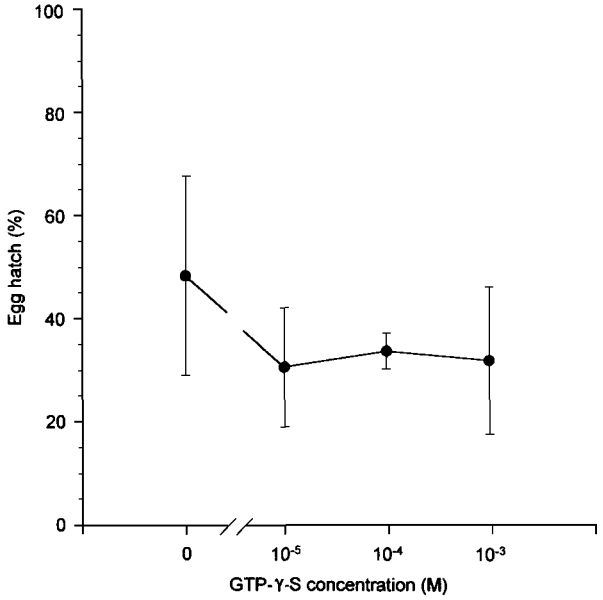

FIG. 2. Effect of GTP-γ-S on EHP-induced egg hatching in *Elminius modestus*. The test egg mass was added to a GTP-γ-S solution containing a 1/16 dilution of partially purified EHP. Results represent means ± SEM ($N = 3$). None of the GTP-γ-S concentrations were significantly different from the control as analyzed by Dunnett's multiple comparisons test (Dunnett, 1955, 1964).

When tested alone, neither GTP nor any of the analogs, induced egg hatching (Table 1). Taken together with the negative result obtained with fluoride ions (Table 1), the data do not support G protein involvement in EHP-induced egg hatching. Assays that examined the potential involvement of calcium channels, cell depolarization (excess K^+), and PKC in signal transduction of egg hatching also proved negative (Table 1).

DISCUSSION

This study provides preliminary pharmacological evidence to suggest that the biochemical mechanism of action of EHP is different from the model of eicosanoid action (Smith, 1989). Notably, no evidence could be obtained to implicate G-protein-linked receptors in the mechanism of action of EHPs. Likewise, assays that examined the involvement of calcium ions (Ca^{2+}) and protein kinase C (PKC) did not support their involvement in egg hatching. Although the

assays were by no means exhaustive, the only putative component of the signal transduction pathway of EHP for which evidence was obtained was cAMP.

The mechanism of action of hydroxylated fatty acids is not well understood. Hydroxyeicosatetraenoic acids (HETEs) are, however, known to incorporate into cell lipids, particularly phospholipids. This has led to the suggestion, by several investigators, that HETEs may act via membrane perturbation effects (Spector et al., 1988). While no data have been obtained in the present study to suggest that this may be the case for EHP, it is nevertheless of interest that a 5% (v/v) ethanol solution induces egg hatching (Clare, unpublished). Ethanol is, of course, well known for causing membrane perturbation effects (e.g., Ordway et al., 1989). On the other hand, 5-HETE has been shown to act by a G-protein-linked mechanism (O'Flaherty and Rossi, 1993). The inhibitory action of 20-HETE [a cytochrome P-450 metabolite of arachidonic acid (Lange et al., 1977)] on K^+ channel activity in renal arteriolar smooth muscle cells is also thought to be G protein dependent (Sun et al., 1997). Unfortunately, EHP has not been identified unequivocally for any species (see Clare, 1997). Assays of hydroxylated fatty acids, however, indicate that the active site is stereospecific. For example, (8*R*)-hydroxyeicosapentaenoic acid [(8*R*)-HEPE] induced hatching of *Elminius modestus* egg masses, whereas 8-(*S*)-HEPE did not (Hill and Holland, 1992). The comparable stereoselectivity of maturation of starfish oocytes by (8*R*)-HETE led Meijer et al. (1986) to suggest that activity may be at the level of the plasma membrane. Clearly, there is a strong possibility that EHP activity is receptor mediated.

Among their actions, hydroxylated fatty acids activate PKC (Hannson et al., 1986; Liu et al., 1991; Lange et al., 1997) and stimulate (O'Flaherty et al., 1988) and inhibit (Smith et al., 1993) an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Neither of these two intracellular messengers was implicated in the mechanism of action of EHP on the basis of the pharmacological experiments reported here. It is suggested that the inability of eggs to hatch in calcium-free seawater reflects a requirement for extracellular calcium for embryo muscular action. Nevertheless, the possibility that EHP needs Ca^{2+} for activity cannot be dismissed. With one exception, compounds that effect increases in intracellular cAMP levels were active when bioassayed. Thus forskolin, which activates adenylate cyclase, and caffeine, theophylline, and IBMX, which inhibit phosphodiesterase, all induced egg hatching. db-cAMP, a lipid permeable, phosphodiesterase-resistant analog of cAMP did not induce egg hatching. This compound has, however, been shown to be without effect in other systems where cAMP is implicated (Jensen and Morse 1990; Clare et al., 1995). The reason for this disparity is unclear.

On the basis of the available evidence, the following working hypothesis for EHP action is suggested: barnacle EHP induces egg hatching via a comparatively simple signal transduction pathway involving modulation by intracellular cAMP. By inference from what is known of other systems, cAMP may subsequently

activate protein kinase A, leading to protein phosphorylation (e.g., Morse, 1992). In arriving at this hypothesis, it needs to be stressed that the evidence against G protein, Ca^{2+}, and PKC involvement is far from unequivocal. There remains the possibility that the barnacle egg case and/or the underlying cuticle of the embryo are relatively impermeable to some of the compounds tested. That said, with the possible exception of gossypol, the pharmacological compounds assayed in the present study have been used for other marine invertebrate larvae to dissect components of signal transduction pathways and, by inference, must be able to cross cell membranes in those species (e.g., Baxter and Morse, 1987; Clare, 1996; Leitz, 1997).

In conclusion, unlike most eicosanoids that act as autocoids, EHP is a relatively stable metabolite that is released by the adult barnacle into seawater to act on the brooded embryos at some distance from its site of release. The mechanism of action of EHP does not appear to involve binding to a G-protein-linked receptor or a dependency on G protein activation. These preliminary data thus support earlier suggestions that not all hydroxy fatty acids accord with the generalized model of eicosanoid action (Spector et al., 1988). For the future, a complete characterization of barnacle (*S. balanoides* and *E. modestus*) EHPs is required so that receptor–ligand interactions and the above working hypothesis can be examined more critically.

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SEX PHEROMONE OF THE COMMON SHEEP MOTH, *Hemileuca eglanterina*, FROM THE SAN GABRIEL MOUNTAINS OF CALIFORNIA

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Abstract—The sex pheromone of *Hemileuca eglanterina* from the San Gabriel Mountains, California, was determined to be a combination of *E*10,*Z*12-hexadeca-10,12-dien-1-yl acetate, *E*10,*Z*12-hexadeca-10,12-dien-1-ol, and *E*10,*Z*12-hexadeca-10,12-dienal. Ratios of the compounds in extracts of female pheromone glands varied around a mean of 100 : 48 : 1.1 of the acetate, alcohol, and aldehyde, respectively. Field trials with synthetic compounds indicated that the optimum ratio of alcohol to aldehyde was 10 : 1 and that this ratio was more critical than the ratio of either compound to the acetate. A synthetic blend of 100 : 10 : 1 acetate–alcohol–aldehyde was effective at attracting male moths in the field. Additional compounds found in both extract and aeration samples failed to significantly increase trap catches of male moths, although some of these minor components elicited responses from male moth antennae in coupled gas chromatography–electroantennography studies.

Key Words—*Hemileuca eglanterina*, *E*10,*Z*12-hexadeca-10,12-dien-1-yl acetate, *E*10,*Z*12-hexadeca-10,12-dien-1-ol, *E*10,*Z*12-hexadeca-10,12-dienal, sex pheromone.

INTRODUCTION

Hemileuca is the most diverse genus in the Saturniidae of the United States and Canada, with 18 species currently recognized (Tuskes et al., 1996). These attractive moths are variable in color, and they have received a great deal of attention

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from collectors. As a result, considerable information on their biology and distribution is available, despite few of the species being of economic importance. Most species are diurnal and locally abundant during their short flight seasons, but they are difficult to catch as they are strong fliers and seldom land.

There have been a number of observations of interspecific cross-attraction (Tuskes, 1984; Tuskes et al., 1996). For example, *H. eglanterina* (Boisduval) males are attracted to females of *H. electra* (Wright) (Tuskes et al., 1996). These two species are not sympatric and do not interact under natural conditions. However, *H. eglanterina* females attract males of *H. nuttalli* (Strecker) (Collins and Tuskes, 1979), a species with which *H. eglanterina* is sympatric throughout Idaho, Nevada, Utah, and portions of several other states, raising the question of how these two species minimize interactions that would result in wasted reproductive effort, particularly as the life-span of the nonfeeding adults is short (a few days).

There are several possible mechanisms that may contribute to reproductive isolation between *H. eglanterina* and *H. nuttalli*. For example, differing diurnal activity patterns were suggested as an isolating mechanism for a population at Monitor Pass, Sonora County, California (Collins and Tuskes, 1979), with males of *H. eglanterina* favoring the morning hours and *H. nuttalli* males flying in late afternoon. These authors also suggested that differing pheromone chemistry may be important because attraction was not symmetrical; *H. nuttalli* males were attracted to *H. eglanterina* females, but not the reverse. The objective of the study reported here was to determine the composition of the sex pheromone of *H. eglanterina*, with the ultimate goal of using this information to investigate pheromone based mechanisms contributing to reproductive isolation between *H. eglanterina* and its sibling species, *H. nuttalli*.

METHODS AND MATERIALS

Insects

Insects were obtained from the Blue Ridge, San Gabriel Mountains, Los Angeles County, California, USA. Larvae were collected from May through July, either on larval hosts [*Prunus emarginata* L., *Symphoricarpos spp.* (Dougl.) Walp., and *Ceanothus* spp. L.], or while wandering before pupation (CAUTION: larvae have stinging spines). Larvae were reared on bouquets of cut host material in screen-covered 40-liter glass aquaria. Mature, wandering larvae were placed in plastic boxes containing peat as a pupation substrate where they were allowed to spin cocoons and pupate. Pupae were kept in screen cages in the shade outdoors to allow natural coordination of emergence. This population requires two years to complete development under natural conditions, but may emerge in one

year if collected as early instars and reared under warmer temperatures (approximately 27°C). Emerging adults were collected daily and were either used for studies immediately or held in 6- × 8-cm screen cages inside sealed plastic bags with a damp towel at 10–15°C for one to two weeks. Cooling was necessary due to the short life-span (less than one week under normal conditions) of the nonfeeding adults. Unmated females generally ceased calling and began depositing infertile eggs within four days of eclosion at 22°C. Virgin females were used for preparation of pheromone extracts, while males were used for electroantennographic studies.

Extraction of Sex Pheromone Glands

Sex pheromone glands were removed from virgin calling females of 1–4 days of age. The gland was extruded by applying gentle pressure to the abdomen near the tip to force eversion of the ovipositor, at which point it and the terminal abdominal segments were excised with small scissors into a 0.25-ml conical glass insert containing ~30 μl pentane. The glands were extracted for 20 min; and then the solvent was transferred to a clean insert, with two rinses with one drop of pentane. Extracts were concentrated as needed by passive evaporation prior to analysis. If not used immediately, extracts were stored at −20°C.

Aeration of Calling Females

Pheromone collection was attempted from three individual calling females (<2 days old) for approximately 90 min in the late afternoon under a combination of natural and fluorescent lighting at 21°C, as follows. A 0.25-ml Kimble glass conical vial insert was modified by grinding off the tip with a diamond grinding wheel until there was a small opening (approx. 1.5–2 mm). A small amount of Porapak Q (precleaned with ether in a Soxhlet extractor) was placed in the narrow end and held in place with ether-extracted glass wool plugs. This collector was inserted into a 1/4-in. brass Swagelok union with a Teflon ferrule, with the other end of the union connected to a vacuum source via a flowmeter (flow 60 ml/min). The chamber consisted of a 10 cm long × 3 cm diameter glass tube tapered at one end to 1/2 in. so that Swagelok fittings could be used to connect it to an air source. A coarse sintered glass frit 5 cm from the open end divided the chamber in half. The air supply side of the chamber was filled with activated charcoal, while the moth was allowed to hang from the sintered glass frit on the other side. An air flow of ~400 ml/min was used to continuously sweep the aeration chamber. The collector was positioned directly below the everted sex pheromone gland of a calling female moth (which could be seen clearly during calling) to sample the moth effluent.

Gas Chromatography

GC analyses were conducted with Hewlett-Packard 5890A or 5890 Series II gas chromatographs (H-P, Palo Alto, California), with helium carrier gas, in splitless mode. Columns and programs used to analyze composite extracts from multiple females included: HP Ultra-1 (H-P; 30 m × 0.25 mm ID, 0.17-μm film, 100°C for 1 min, then 10°C/min to 275°C, hold for 45 min), DB-225 (J&W Scientific, Folsom, California; 30 m × 0.26 mm ID, 0.25-μm film, 100°C for 1 min, then 10°C/min to 220°C, hold for 45 min), DB-1701 (30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, then 5°C/min to 275°C, hold for 45 min), and DB-Wax (30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, then 10°C/min to 250°C, hold for 45 min). Extracts from individual females were analyzed on a DB-5 column (30 m × 0.32 mm ID, 0.25-μm film, programmed as for the H-P Ultra-1 column above). Tentative identifications were made by comparison of compound retention times in extracts to those of synthetic standards.

Coupled Gas Chromatography-Electroantennography (GC-EAD)

Aliquots of female extracts, aerations, and synthetic standards were analyzed by splitless coupled GC-EAD, with an H-P 5890 series II GC equipped with a DB-5 column (30 m × 0.32 mm ID, 0.25-μm film; J&W Scientific), programming from 100°C at 0 min, then 10°C/min to 250°C, and holding for 20 min, and with an injector temperature of 250°C. The column effluent was split equally with a press-fit Y-connector (J&W Scientific) into two branches of 0.25 mm uncoated fused silica tubing, with one branch going to the GC's flame ionization detector (FID), and the other being directed through a heated transfer line (275°C) to a 1-cm-diameter glass stimulus delivery tube. The capillary effluent was diluted with humidified air (400 ml/min) and passed over the male moth antennal preparation (see below). The moth antenna was suspended between glass capillary electrodes filled with insect physiological saline (modified Locke's saline, 7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1 liter distilled water) (Humason, 1972), with electrical contact to the custom-built multistage amplifier made with AgCl-coated silver wires. The amplifier and FID outputs were simultaneously recorded on a matched pair of H-P 3394 recording integrators.

Male antennae were removed by using fine forceps to firmly hold the scape and pull the antenna free of the head without crushing the scape. The terminal rami and tip of the antenna were removed so that the end of the antenna could be placed into the saline-filled recording electrode. The antenna was positioned on the amplifier mount at the end of the delivery tube such that the plane of the antennal branches was perpendicular to the airflow from the stimulus delivery tube. A single antennal preparation could be used for several hours with periodic additions of saline.

Coupled Gas Chromatography–Mass Spectrometry

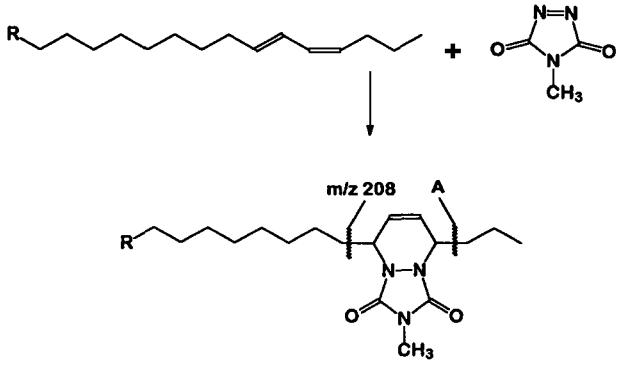
EI mass spectra (70 eV) were taken with an H-P 6890 gas chromatograph interfaced to a 5973 mass selective detector. A 30 m × 0.25 mm ID HP-5MS column was used (H-P), programmed at 50°C for 1 min, then 10°C/min to 250°, and holding for 20 min. Compounds in the insect extract were identified by comparison of retention times and mass spectra with those of authentic standards (see below). A composite sample from five females obtained in July 1997 was analyzed.

Formation of Adducts with 4-Methyl-1,2,4-triazoline-3,5-dione (MTAD) (Scheme 1)

A combined extract from five pheromone glands was concentrated to ~5 μl, then diluted with 20 μl of CH_2Cl_2. A 10-μl aliquot was transferred to a Kimble 0.25-ml glass conical vial insert and treated with 2.5 μl of a solution of MTAD in CH_2Cl_2 (1.2 mg/ml). The faint pink solution was concentrated under a gentle stream of nitrogen to <5 μl, and a 1-μl aliquot was analyzed by splitless GC-MS (injector 300°C, temperature program: 100°C at 0 min, then 15°C/min to 300°C for 20 min; solvent delay was 8 min; instrument and column are as described above).

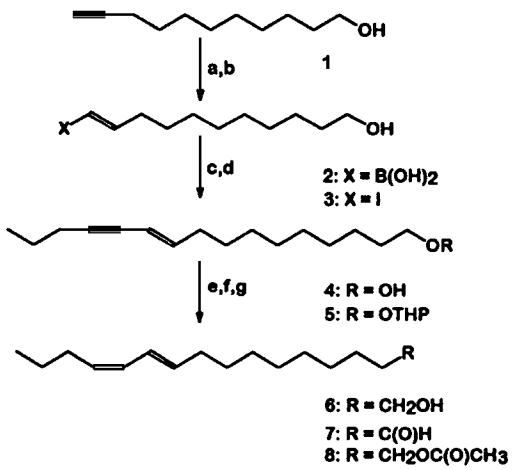
Synthesis of Pheromone Components

Proton NMRs were recorded on a General Electric QE 300 instrument (300 MHz) in $CDCl_3$ solution. EI mass spectra (70 eV) were taken with an H-P



R = CH_2OAc, A = m/z 350
R = CH_2OH, A = m/z 308
R = CHO, A = m/z 306

SCHEME 1.



a) i. Catecholborane, ii. water; b) NaOH, I_2; c) Pentyne, Pd^{+2}, CuI, diisopropylamine; d) Dihydropyran, H^+; e) i. Dicyclohexylborane, ii. AcOH, iii. MeOH, H^+; f) PDC; g) AcCl, pyridine

SCHEME 2.

5970B mass selective detector interfaced to a 5890 GC fitted with a DB5-MS column (20 m × 0.2 mm ID, J&W Scientific). Air- and/or water-sensitive reactions were carried out in oven-dried glassware under an N_2 atmosphere. Tetrahydrofuran (THF) was purified by distillation from sodium–benzophenone ketyl under argon. Flash chromatography was carried out with 230–400 mesh silica gel (Aldrich Chemical, Milwaukee, Wisconsin).

Synthesis of E10,Z12-Hexadeca-10,12-dien-1-ol (E10,Z12–16:OH) (Scheme 2).

(E1)-1-Iodoundecen-11-ol (3). A mixture of 10-undecyn-1-ol **1** (4.9 g, 29.2 mmol; Farchan Labs, Gainesville, Florida), catecholborane (7.0 ml, 66 mmol; Aldrich), and THF (10 ml) were stirred at 70°C overnight under N_2. After cooling to room temperature, 180 ml water was added, and the mixture was stirred 4 hr. The resulting white slurry was cooled in an icebath, filtered, and the solids rinsed three times with 50 ml icewater. The sticky white boronic acid **2** (5.83 g) was dried under aspirator vacuum and used without further purification. NMR: δ 5.78 (br. d, 1H, 18 Hz), 5.42 (br. d, 1H, J = 18 Hz), 3.64 (t, 2H, J = 6.5 Hz), 2.15 (m, 2H), 1.6–1.2 (m, 14H).

The dried (*E*1)-11-hydroxy-1-undecenylboronic acid **2** (2.9 g, 13.7 mmol) was taken up in a mixture of ether (40 ml) and 1.5 M NaOH (60 ml) and stirred vigorously for 30 min. The mixture was cooled to 0°C, and iodine (3.82 g, 15

mmol) in 15 ml ether was added dropwise over 30 min. The mixture was stirred an additional 30 min at 0°C, then quenched by addition of a few mls of saturated aq. Na_2SO_3. The layers were separated and the aqueous phase extracted with hexane. The combined organic phases were washed with dilute aq. Na_2SO_3 and brine and then concentrated and purified by flash chromatography (22 × 5 cm), eluting with 22.5% EtOAc in hexanes, yielding 2.6 g of iodoalcohol **3** as a pale yellow oil, which solidified upon refrigeration. NMR: δ 6.51 (dt, 1H, J = 14.4, 7.0 Hz), 5.98 (br. d, 1H, J = 14.2 Hz), 3.64 (t, 2H, J = 6.5 Hz), 2.05 (br. quart, 2H, J = 6.8 Hz), 1.55 (m, 2 H), 1.5–1.22 (m, 12H). MS: m/z 180 (31), 167 (23), 109 (28), 95 (77), 81 (53), 67 (66), 55 (89), 41 (100).

E10-Hexadecen-12-yn-1-ol (4). To a dry 250-ml three-neck flask under N_2 were added sequentially 0.25 g bis(triphenylphosphine)palladium(II) chloride, 0.25 g CuI, iodoalcohol **3** (2.5 g, 8.4 mmol), and 1-pentyne (1.36 g, 20 mmol) in 25 ml of THF. Diisopropylamine (3 g) was then added dropwise with stirring, and the reaction mixture warmed and precipitated brown–black solids. The slurry was stirred for 1 hr, then hexane (100 ml) was added, and after stirring for 5 min, the mixture was filtered with suction. The brown solution was washed twice with sat. aq. NH_4Cl, dried, and concentrated. MS: m/z 236 (3), 208 (1), 207 (1), 193 (2), 179 (4), 121 (12), 107 (18), 94 (21), 93 (37), 91 (45), 79 (100), 67 (29), 55 (34), 41 (44).

E10,Z12-Hexadeca-10,12-dien-1-ol (6). The crude enynol **4** was taken up in ether, treated with 2 ml dihydropyran and a few crystals of *p*-toluenesulfonic acid (PTSA), and the mixture stirred overnight. The ether solution was then washed twice with dilute $NaHCO_3$, dried, concentrated, and purified by flash chromatography (20 × 5 cm) eluting with 7.5% EtOAc in hexanes, yielding 2.1 g of the protected alcohol **5** as a yellow oil. NMR: δ 6.05 (dt, 1H, J = 15.8, 7.1 Hz), 5.46 (dt, 1H, J = 16.0, 1.6 Hz), 4.58 (br t, 1H, J ~ 4.6 Hz), 3.87 (m, 1H), 3.74 (dt, 1H, J = 10.5, 6.5 Hz), 3.50 (m, 1H), 3.38 (dt, 1H, J = 9.6, 6.6 Hz), 2.27 (td, 2H, J = 7.1, 1.8 Hz), 2.07 (br. quart, 2H, J ~ 6.9 Hz), 1.9–1.47 (m, 10 H), 1.43–1.22 (m, 12H), 0.98 (t, 3H, J = 7.5 Hz).

Cyclohexene (2.0 ml, 20 mmol) was added dropwise over 10 min to a solution of borane–dimethylsulfide complex (1 ml, 10 mmol) in THF (12 ml) at 0°C under N_2. The mixture was warmed to room temperature, stirred 2 hr, cooled again to 0°C, and the crude enynol THP ether **5** (2 g, ~6.3 mmol) was added dropwise. The solution was allowed to warm slowly to room temperature, monitoring the disappearance of **5** by TLC. The mixture was cooled to 0°C and glacial acetic acid (4 ml) was added dropwise. It was warmed to room temperature and stirred overnight, then cooled to 0°C in a Dry Ice–acetone bath while 5 M NaOH (15 ml) and 30% H_2O_2 were added sequentially (EXOTHERMIC!). The mixture was diluted with water and extracted with pentane. The pentane extracts were dried and concentrated, taken up in MeOH (50 ml) with a few crystals of PTSA, and stirred overnight to remove the THP group. The mixture was worked up by

addition of 1 g $NaHCO_3$, removal of most of the MeOH by rotary evaporation, and partitioning of the residue between water and hexane. The hexane extracts were washed with brine, dried, concentrated, and purified by flash chromatography, yielding 1.04 g of *E*10,*Z*12-hexadecadienol **6**, >98.9% pure by GC. NMR: δ 6.31 (ddt, 1H, J = 14.3, 11.1, 1.1 Hz), 5.97 (br. t, 1H, J = 10.8 Hz), 5.66 (dt, 1H, J = 15.0, 7.0 Hz), 5.32 (dt, 1H, J = 10.8, 7.6 Hz), 3.65 (t, 2H, J = 6.6 Hz), 2.2–2.05 (m, 4H), 1.62–1.25 (m, 16H), 0.93 (t, 3H, J = 7.4 Hz). MS: m/z 238 (11), 149 (2), 135 (5), 121 (8), 109 (15), 96 (30), 95 (29), 82 (36), 81 (50), 79 (32), 67 (100), 55 (37), 41 (49).

E10,Z12-Hexadeca-10,12-dienal (E10,Z12–16:Ald) (7). Dienol **6** (0.44 g, 1.85 mmol) was stirred overnight with pyridinium dichromate (1.1 g, 3 mmol) in 5 ml CH_2Cl_2. The mixture was poured into 50 ml hexane, stirred for 5 min, then filtered. After concentration, the residue was flash chromatographed (4% ether in hexane) and Kugelrohr distilled (oven temp. ~120°C, 0.07 mm Hg), yielding the pure aldehyde. The NMR and mass spectral data closely matched literature values (Samain and Descoins, 1979; Bestmann et al., 1981). The final product was diluted in heptane to a concentration of 10 mg/ml to inhibit trimerization, BHT was added as a antioxidant, and the solution was stored at −20°C until needed.

E10,Z12-Hexadeca-10,12-dien-1-yl Acetate (E10,Z12–16:Ac) (8). Acetyl chloride (0.5 ml) was added dropwise to a solution of dienol **6** (150 mg, 0.6 mmol) and pyridine (1 ml) in dry ether (50 ml), and the mixture was stirred overnight at room temperature. Then 0.5 M $NaHCO_3$ (50 ml) was added and the mixture stirred for 15 min. The layers were separated, and the aqueous phase was extracted with hexane. The combined organic phase was washed with 0.1 M HCl and brine, dried, concentrated, and Kugelrohr distilled (oven 150°C, 0.07 mm Hg), yielding 160 mg of the acetate. The NMR and mass spectral data agreed with literature values (Samain and Descoins, 1979; Bestmann et al., 1981), and the GC and MS data perfectly matched those obtained from an authentic standard obtained from Darwin Reed (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada).

10,12-16:OH Isomers. Aliquots (80 mg) of a sample of the mixed geometric isomers of 10,12–16:OH, generated by scrambling the double bonds of *E*10,*Z*12–16 : OH with aqueous $NaNO_2$ and HNO_3 (Sonnet, 1974), were separated by passage through a 1.2-m × 6-mm-ID column of macroporous ion exchange resin (Lewatit SP 1080, Ag^+ form, E. Merck, Gibbstown, New Jersey), eluting with MeOH (1.5 ml/min) (Houx et al., 1974) and monitoring the effluent with a Dynamax RI-2 refractive index detector (Rainin Instruments, Emeryville, California). Fractions that were >99% isomerically pure were combined, concentrated, and taken up in hexane. The hexane solutions were dried over anhyd. Na_2SO_4, then concentrated and Kugelrohr distilled (oven temp. ~155°C, 0.03 mm Hg).

Field Trials

Field trials were conducted at the Blue Ridge site where larvae had been obtained during July and August of 1993 through 1996 (see above). Initially, Pherocon 1C traps were used (Trécé Inc., Salinas, California), but these proved inadequate due to the large size of the moths and the numbers attracted. Consequently, traps of our own design were used, consisting of a 150-mm-diameter plastic funnel (1 pint funnel, No. 5016, Contico Automotive Corp., St. Louis, Missouri) resting upon a 3.2-mm-mesh wire screen cylinder (14.5–15 cm OD by 20–25 cm high). The spout was removed from the funnel, leaving a 45- to 50-mm opening at the bottom. A 32-gauge wire was wrapped around a tab on the lip of the funnel and bent so that the lure could be suspended over the center of the funnel. The wires at the bottom of the screen cylinder were bent inward so that a 150-mm plastic Petri dish could be rested inside the cylinder as a bottom and weighted with rocks or soil for stability.

Lures consisted of 11-mm red rubber septa (Wheaton Scientific, Millville, New Jersey), individually labeled with a treatment number and loaded with heptane solutions (100 μl) of test compounds, plus two drops of 10 mg/ml butylated hydroxytoluene (BHT) in heptane as an antioxidant. Once the treatment solutions had soaked into the septa, the lures were impaled on No. 6 bronzed fishhooks and stored in 20-ml glass vials for transport. Lures were kept in a freezer or an ice-chest when not in use. No one set of lures was used for more than five days.

A single trial consisting of three blocks located at different points along the ridge. Traps were spaced approximately 10 m apart, and blocks were 600 m or more apart. Two to five trap counts were made at one-day intervals, with the traps being cleaned out and rerandomized after each reading. Counts were made early in the morning or, more often, late in the day after flight had ceased.

Statistics

For the statistical analysis of field trials, elimination of the effect of day was accomplished by pooling the day counts for each treatment in a given block. This sum was then transformed ($\sqrt{x} + 0.5$) prior to conducting a two-way ANOVA, followed by linear and quadratic contrasts. If significant effects were detected, then the Student-Neuman-Keuls (SNK) test was used to separate means (SAS Institute 1996).

RESULTS

Analysis of Female Extracts. Analysis of female pheromone gland extracts by coupled GC-EAD demonstrated that male moth antennae responded strongly to three compounds, with smaller, less consistent responses to several other com-

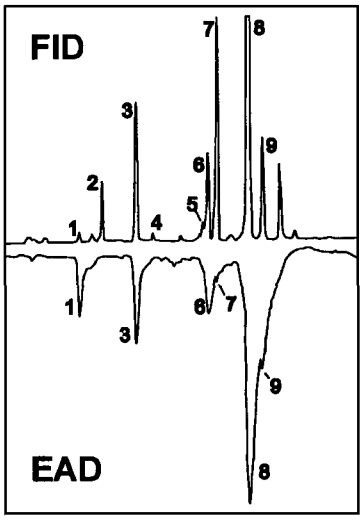


FIG. 1. Coupled gas chromatogram–electroantennogram of male *H. eglanterina* (Boisduval) antenna stimulated by a female extract. Column used was DB-5 (30 m × 0.32 mm ID, 0.25-μm film, program used was 100°C for 1 min, then 10°C/min to 275°C, held for 45 min). Identifications of peaks: 1, *E*10,*Z*12–16 : Ald; 2, 16 : OH; 3, *E*10,*Z*12–16 : OH; 4, *E*10,*E*12–16 : OH; 5, *Z*10–16 : Ac; 6, *E*- and *Z*11–16 : Ac; 7, 16 : Ac; 8, *E*10,*Z*12–16 : Ac; and 9, *E*10,*E*12–16 : Ac.

ponents (Figure 1). These compounds, and several others present in the extracts, were identified by GC and GC-MS (Table 1). The mass spectrum of the most abundant of the antennal stimulatory compounds (and the component eliciting the strongest antennal response) was consistent with that of a conjugated 16-carbon diene acetate, with a comparatively strong molecular ion (m/z 280, 20% of base peak, possible molecular formula $C_{18}H_{32}O_2$), and a small but distinct fragment at m/z 220 (2%, loss of acetic acid). Furthermore, the spectrum closely matched a database spectrum of *E*10,*Z*12–16 : Ac (NIST/EPA/NIH Mass Spectral Database 1992). The compound from the extract was confirmed to be *E*10,*Z*12–16 : Ac in several ways. First, the position of the conjugated diene system was confirmed by formation of the adduct with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) (Young et al., 1990). The EI mass spectrum of the resulting derivative gave a molecular ion at m/z 393 (5%), and diagnostic fragments at m/z 350 (19%) and 208 (100%) from cleavage of the alkyl groups on the carbons α to the two nitrogens (Scheme 1), unequivocally fixing the diene in the 10,12 position. Second, the *E*10,*Z*12 stereochemistry was unambiguously proven by comparison of retention times on five columns of differing polarity (HP Ultra-1, DB5-MS, DB-1701, DB-225, DB-Wax) with those of synthetic

TABLE 1. COMPOUNDS, METHODS OF IDENTIFICATION, AND AMOUNTS RELATIVE TO *E*10,*Z*12–16 : Ac IN EXTRACTS AND AERATIONS OF FEMALE *Hemileuca eglanterina* FROM SAN GABRIEL MOUNTAINS

Compound	Identification methods used[a]		Amount in single female extracts relative to *E*10,*Z*12–16 : Ac[b]				Amount in	
	GC	GC-MS	Mean	SD	Range	% detected[c]	Combined extract[d]	Aeration
*E*10,*Z*12–16 : Ac	X	X	100			100	100	100
*E*10,*Z*12–16 : OH	X	X	48.4	52.5	6.0–260	96	30	30
*E*10,*Z*12–16 : Ald	X	X	1.1	0.8	0.3–3.3	56	0.9	
*E*10,*E*12–16 : Ac	X	X	10.4	2.9	4.8–15.1	100	7.4	4.3
*E*10,*E*12–16 : OH	X	X	4.6	1.0	1.7–21.8	37	1.4	
E/*Z*11–16 : Ac	X	X	10.8	4.9	5.2–25.2	93	8.8	
*Z*10–16 : Ac	X		2.1	0.7	1.2–3.1	22	1.6	
16 : Ac	X	X	53.2	43.9	16.4–200	100	35	114
16 : OH	X	X	57.5	71.6	4.1–285	96	11	

[a]GC = retention time matches on at least three columns (see materials and methods) with authentic standards. GC-MS = Mass spectral match with authentic standards.
[b]Means of 27 female extracts.
[c]Percent of female extracts with detectable amounts.
[d]Combined extract from six females.

standards. Standards of the other three 10,12–16 : Ac isomers (synthesized by and obtained from D. Reed, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada), had retention times different from *E*10,*Z*12–16 : Ac on four of the five GC columns (Table 2). Furthermore, GC-EAD of the isomers of 10,12–16 : Ac indicated that *E*10,*Z*12–16 : Ac elicited the largest response from male antennae (mean mV ± SD of 10 males tested *EZ*, 0.66 ± 0.17; *ZE*, 0.39 ± 0.10; *ZZ*, 0.28 ± 0.06; and *EE*, 0.26 ± 0.07; one-way ANOVA, $F = 24.4$, $df = 39$, $P < 0.0001$ followed by SNK, $\alpha = 0.05$).

The second EAD-active compound (Figure 1, peak 3) gave a comparatively strong molecular ion at *m*/*z* 238 (16% of base peak at *m*/*z* 67), 42 amu less than *E*10,*Z*12–16 : Ac, and consistent with the corresponding alcohol, *E*10,*Z*12–16 : OH. This tentative identification was confirmed from the mass spectrum of the MTAD adduct [molecular ion at *m*/*z* 351 (9%), and diagnostic fragments at *m*/*z* 208 (100%) and 308 (36%), Scheme 1]. The geometry of the diene was confirmed by retention time comparisons with synthetic standards of the four isomers on five columns. Furthermore, the mass spectrum exactly matched that of an authentic standard of *E*10,*Z*12–16 : OH.

The third compound eliciting consistent EAD activity, while being present in only small amounts (Figure 1, peak 1), was identified by combination of several data. First, the selected ion monitoring profile of eight ions (236, 109, 96, 95, 82, 81, 67, 55) and the retention time matched those of an authentic stan-

TABLE 2. RETENTION TIMES (MINUTES) OF ISOMERS OF 10,12–16 : Ald, 10,12–16 : OH, AND 10,12–16 : Ac ON CAPILLARY GC COLUMNS OF DIFFERING POLARITY[a]

Compound and isomer	Columns (increasing polarity →)				
	Ultra-1	DB-5	DB-1701	DB-225	DB-Wax
(10,12)–16 : Ald					
ZE	9.30	10.70	13.14	12.63	11.37
EZ	9.40	10.79	13.25	12.76	11.52
ZZ	9.51	10.92	13.35	12.85	11.63
EE	9.57	10.97	13.39	12.83	11.73
(10,12)–16 : OH					
ZE	10.02	11.37	13.83	13.46	13.53
EZ	10.11	11.45	13.92	13.56	13.64
ZZ	10.22	11.58	14.04	13.68	13.77
EE	10.28	11.62	14.07	13.65	13.85
(10,12)–16 : Ac					
ZE	11.31	12.66	14.79	13.77	12.81
EZ	11.40	12.74	14.88	13.87	12.94
ZZ	11.51	12.87	14.99	13.98	13.06
EE	11.57	12.91	15.02	13.95	13.15

[a]Temperature programs are described in Materials and Methods.

dard. Second, although the mass spectrum of the MTAD adduct did not show a molecular ion, it clearly showed diagnostic fragments at m/z 306 and 208, confirming the 10,12 position of the diene. Third, in retention time comparisons, only the *E*10,*Z*12–16 : Ald isomer of the four 10,12–16 : Ald isomers matched the retention time of the insect produced compound on all five GC columns (Table 2).

Confirmation of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald as the correct structures of the pheromone components was obtained from the strong antennal responses elicited from male moth antennae in coupled GC-EAD experiments and from the results of field trials (see below).

In addition to these three compounds, several other components in the pheromone gland extracts also elicited small responses from male antennae in some GC-EAD runs or were present is significant amounts (Figure 1, Tables 1 and 3). These compounds included 16 : OH, 16 : Ac, *E*10,*E*12–16 : Ac, *E*10,*E*12-16 : OH, *Z*10–16 : Ac, plus *E*- and *Z*11–16 : Ac, all of which were identified by comparison of retention times on multiple GC columns, and, with the exception of the *Z*10–16 : Ac, by comparison of mass spectra. For identification of the monoene acetates, the retention times were measured on DB-Wax of the series of *E* and *Z* isomers of monoene hexadecenyl acetates in which the double-bond position ranged from carbon 5 to 13. Comparison of retention times narrowed

TABLE 3. ELECTROANTENNOGRAPHIC RESPONSES (RELATIVE TO *E*10,*Z*12–16 : Ac) AND PERCENT OF MALE *Hemileuca eglanterina* ANTENNAE RESPONDING TO COMPOUNDS IN FEMALE EXTRACTS AND AERATION[a]

	Response to female extracts[b]			Aeration	
Compound	mean (%)	range (%)	% detected	Mean	SD
*E*10,*Z*12–16 : Ac	100		100	100	
*E*10,*Z*12–16 : OH	35	13–81	88	14	[c]
*E*10,*Z*12–16 : Ald	15	4–28	81	8	2.6
*E*10,*E*12–16 : Ac	43	21–56	50	50	18.1
E/*Z*11–16 : Ac	21	6–60	94	14	2.3
16 : Ac	17	12–31	31	11	[c]

[a]Percent of female extracts that elicited responses by male antennae.
[b]Responses from 10 males.
[c]Only detected by one of the two males used for the aeration sample.

the possibilities down to a few, one of which was *Z*11–16 : Ac. This assignment was corroborated by coinjection of *Z*11–16 : Ac with a composite female extract, resulting in an exact retention time match. Furthermore, using *Z*11–16 : Ac and 16 : Ac as references, comparison of retention times on multiple columns indicated that *Z*10–16 : Ac was present as well, because the relative areas of the *Z*11–16 : Ac to the *Z*10–16 : Ac peaks in the female extract were different on the two different columns, with the ratio being 2.8 : 1 on DB-Wax and 5.5 : 1 on DB-5. This discrepancy in ratios can be explained by the fact that on DB-Wax, *Z*10- and *E*11–16 : Ac coelute, whereas on DB-5, *E*11- and *Z*11–16 : Ac coelute. Thus, on DB-Wax, the *Z*10–16 : Ac peak is actually a composite with *E*11–16 : Ac and the *Z*11–16 : Ac peak is due to the single compound, while on DB-5, the situation is reversed, with the *Z*10–16 : Ac peak arising from a single component, and the *Z*11–16 : Ac peak now being a composite with *E*11–16 : Ac. Thus, all three isomers (*Z*11–16 : Ac, *Z*10–16 : Ac, and *E*11–16 : Ac), were tentatively determined to be in female extracts.

Only one of the three female aerations yielded detectable amounts of pheromone. GC peaks were obtained for *E*10,*Z*12–16 : Ac, *E*10,*E*12–16 : Ac, 16 : Ac, and *E*10,*Z*12–16 : OH in a 100 : 4.3 : 114 : 30 ratio (Table 1). Although the very small *E*10,*Z*12–16 : Ald and *E*/*Z*11–16 : Ac peaks were not integrated by the GC integrator, they were detected by EAD (Table 2), confirming that the compounds found in female gland extracts are actually released by calling females and that the ratios in the aeration samples were consistent with those found in gland extracts.

Of all the compounds from pheromone gland extracts or aeration samples that elicited GC-EAD responses, *E*10,*Z*12–16 : Ac was consistently the most abundant compound and elicited the largest responses from male anten-

nae. Estimates of the total amounts of *E*10,*Z*12–16 : Ac present in single female extracts ranged from 4 to 229 ng with a mean of 57 ng. For *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald, the mean ratio for 27 female extracts was 100 : 48 : 1.1 respectively (Table 1). For the remaining compounds identified (*E*10,*E*12–16 : Ac, *E*10,*E*12–16 : OH, *E*/*Z*11–16 : Ac, Z10–16 : Ac, 16 : Ac, and 16 : OH), the mean ratio was 10 : 2 : 11 : 2 : 53 : 58 respectively (expressed as a percentage of the *E*10,*Z*12–16 : Ac) for the 27 single female extracts (Table 1). Thus, *E*10,*Z*12–16 : Ac was considered the major component of the pheromone when optimizing the blend ratio in field trials, with the other components expressed as a percentage of this compound.

Field Trials. The first field trial tested the three most EAD-active compounds (*E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald) singly, in all binary combinations at two ratios (1 : 1 and 10 : 1), and in a ternary blend mimicking the blend found in a female gland extract (Figure 2). All traps baited with single components caught no moths, while several of the binary blends attracted a few moths. All baits that attracted moths contained *E*10,*Z*12–16 : Ac, indicating its role as a crucial component of the blend, whereas the roles of the other two components were less clear. None of the binary blends were as attractive as the three component blend (Figure 2), suggesting that all three compounds were necessary for optimal attraction.

Because of the short flight season of this species, optimization trials of the

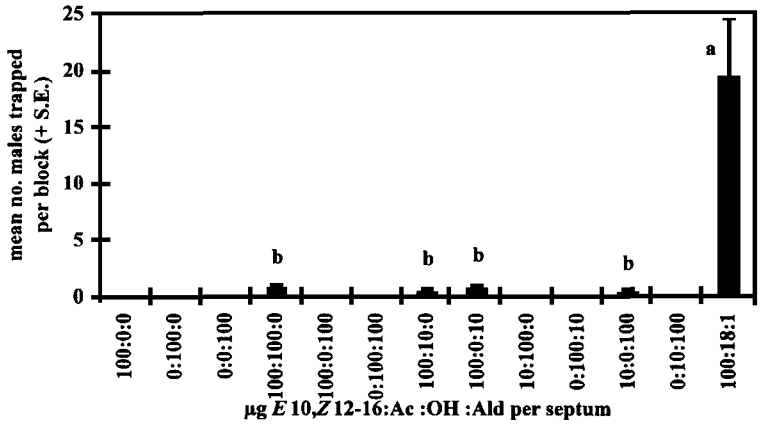


FIG. 2. Results of *Hemileuca eglanterina* (Boisduval) field trials of potential pheromone components as single components, binary blends at two different rates, and a ternary blend. The trial was conducted from July 20 to 22, 1993. Total number of moths trapped was 64. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for blend effect, $F = 20.6$; $df = 4, 8$; $P = 0.0003$.

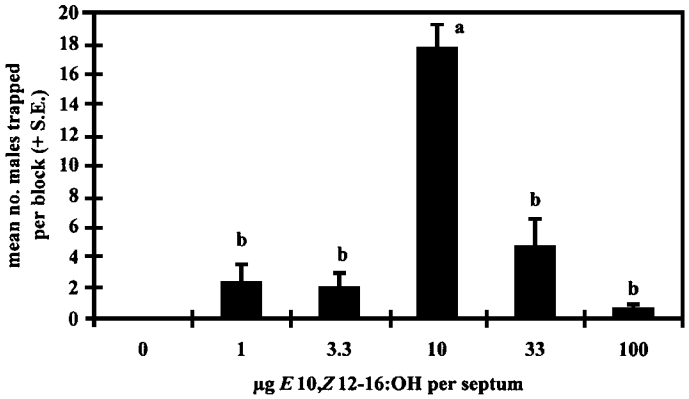


FIG. 3. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the *E*10,*Z*12–16 : OH rate, with *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : Ald held constant at 100 and 1 μg/septum, respectively. The trial was conducted from July 27 to 30 and August 3 to 4, 1993. Total number of moths trapped was 82. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : OH rate effect, $F = 10.5$; $df = 4, 8$; $P = 0.0028$; linear contrast, $F = 0.05$; $df = 1$; $P = 0.8286$; quadratic contrast, $F = 22.6$; $df = 1$; $P = 0.0014$.

ratios of *E*10,*Z*12–16 : Ald and *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ac were conducted concurrently. In each of the two tests, the dose of *E*10,*Z*12–16 : Ac was held constant (100 μg/septum), and either *E*10,*Z*12–16 : Ald or *E*10,*Z*12–16 : OH was added in varying amounts, bracketing the amounts found in female pheromone gland extracts. For the series with varying amounts of *E*10,*Z*12–16 : Ald, the *E*10,*Z*12–16 : OH was held constant at 33 μg/septum, while in the trial with varying amounts of *E*10,*Z*12–16 : OH, *E*10,*Z*12–16 : Ald was held constant at 1.0 μg/septum.

For the *E*10,*Z*12–16 : OH rate series, the 100 : 10 : 1 blend of *E*10,*Z*12–16 : Ac to *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald was most attractive (Figure 3). For the *E*10,*Z*12–16 : Ald rate test, the 100 : 33 : 3.3 rate attracted the most moths, although it was not statistically significantly better than the 100 : 33 : 10 or 100 : 33 : 1 blends (Figure 4). Comparing the best blends from each series, the 100 : 10 : 1 blend was significantly better than the 100 : 33 : 3.3 blend (*t* test, $t = 2.21$, $df = 22$, $P = 0.038$).

The fact that the two best blends both used a 10 : 1 ratio of the *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald suggested that the ratio of these minor components to each other might be more important than the ratio of either component to the major component, *E*10,*Z*12–16 : Ac. Two further tests were conducted to address this question. In the first, the *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald were held constant at 10 and 1 μg/septum, respectively, while

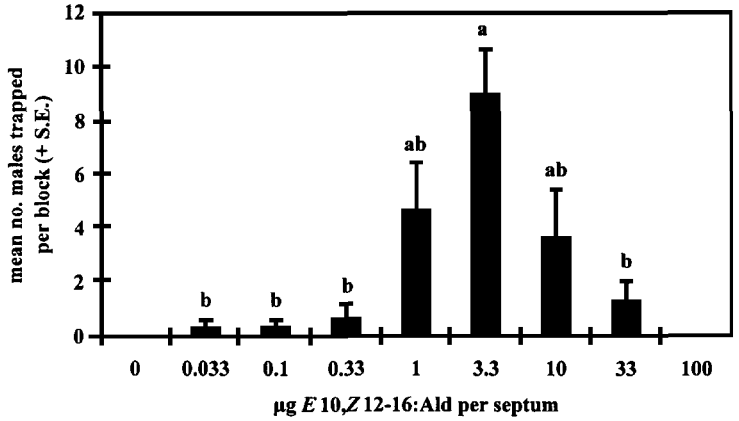


FIG. 4. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the *E*10,*Z*12–16 : Ald rate, with *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : OH held constant at 100 and 33 µg per septum, respectively. The trial was conducted from July 27 to 30 and August 3 to 4, 1993. Total number of moths trapped was 60. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ald rate effect, $F = 5.5$; $df = 6, 12$; $P = 0.0058$; linear contrast, $F = 8.2$; $df = 1$; $P = 0.0145$; quadratic contrast, $F = 10.0$, $df = 1$; $P = 0.0081$.

the dose of *E*10,*Z*12–16 : Ac was varied. The results indicated that increasing the amount of *E*10,*Z*12–16 : Ac in the blend increased the attractiveness of the lure (Figure 5), suggesting that the ratio of the major component to the minor components was not as important as its absolute dose.

To approach this from a different perspective, a second trial was conducted in which *E*10,*Z*12–16 : Ac was held constant and the amount of the two lesser components was varied, while keeping the ratio of those two components constant at 10 : 1. The 100 : 10 : 1 ratio attracted the most moths, but there was no significant difference between this treatment and a number of others, spanning more than a 100-fold range of ratios (Figure 6).

The final field trial addressed the roles of the other pheromone gland components that were present in extracts in significant amounts and/or that elicited small antennal responses in some GC-EAD replicates. In a subtractive bioassay of attraction, in which a "complete" reconstruction of the blend from the gland was compared to blends with single component deletions (Figure 7), only elimination of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, or *E*10,*Z*12–16 : Ald resulted in significantly lower trap catches than the complete blend. Thus, there was no statistical evidence that these other components either synergize or antagonize the response to the three major components.

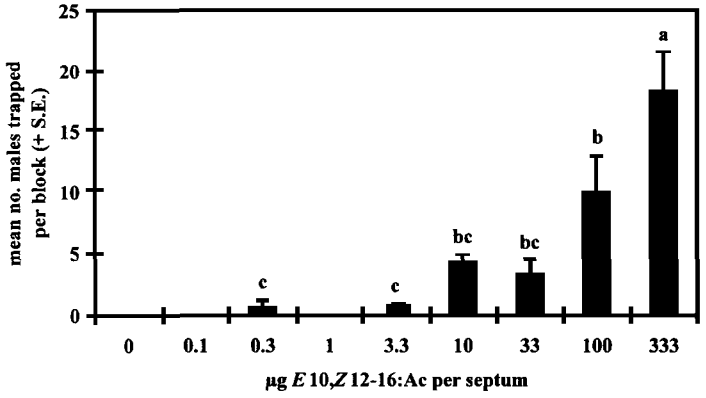


FIG. 5. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the *E*10,*Z*12–16 : Ac rate, with *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald held constant at 10 and 1 µg/septum, respectively. The trial was conducted from July 10 to 13, 1994. Total number of moths trapped was 112. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ac rate effect, $F = 14.3$; $df = 5, 10$; $P = 0.0003$; linear contrast, $F = 63.2$; $df = 1$; $P = 0.0001$; quadratic contrast, $F = 3.8$; $df = 1$; $P = 0.0792$.

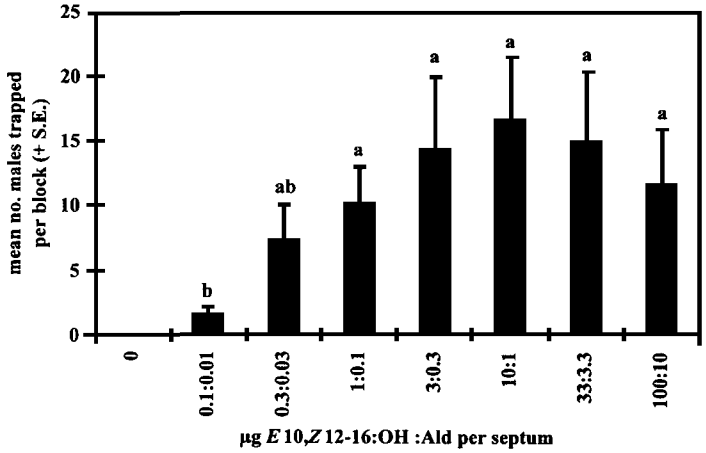


FIG. 6. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the minor components, when altered in tandem. The *E*10,*Z*12–16 : Ac rate was held constant at 100 µg/septum, while the combined dose of *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald, held at a 10 : 1 ratio, was varied. The trial was conducted from July 26 to 28, 1994. Total number of moths trapped was 230. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : OH/*E*10,*Z*12–16 : Ald rate effect, $F = 4.4$; $df = 6, 12$; $P = 0.0146$; linear contrast, $F = 15.6$; $df = 1$; $P = 0.0019$, quadratic contrast, $F = 10.1$; $df = 1$; $P = 0.0080$.

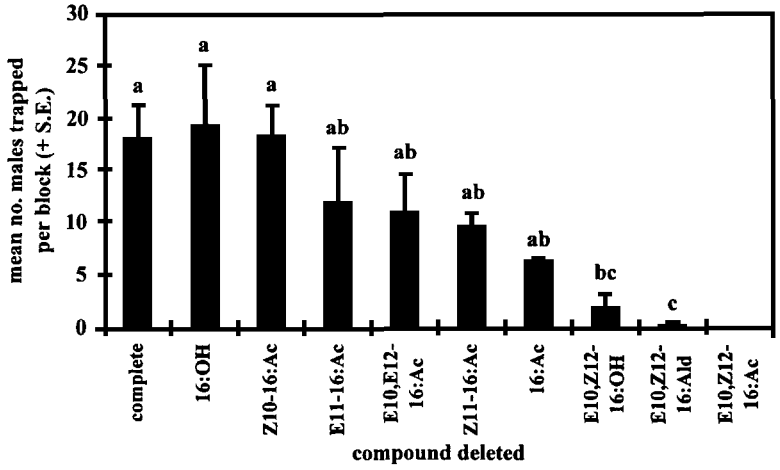


FIG. 7. Attractiveness to male *Hemileuca eglanterina* (Boisduval) of a reconstructed blend mimicking that obtained from female pheromone gland extracts versus blends containing single deletions from a complete blend. Amounts of materials (μg/septum): *E*10,*Z*12–16 : Ac, 100; *E*10,*Z*12–16 : OH, 30; *E*10,*Z*12–16 : Ald, 1; *E*10,*E*12–16 : Ac, 8; *Z*10–16 : Ac, 1.7; *Z*11–16 : Ac, 8.3; *E*11–16 : Ac, 1; 16 : Ac, 33; and 16 : OH, 11. The trial was conducted from July 21 to 24, 1996. Total number of moths trapped was 289. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for additive effect, $F = 7.3$; $df = 8, 16$; $P = 0.0004$.

DISCUSSION

The MTAD adducts proved to be useful in the identification of the three major pheromone components, for several complimentary reasons. First, the adducts were easy to form in quantitative yield by simply treating the pheromone extract with an aliquot of MTAD in CH_2Cl_2. Second, the increased molecular weight of the resulting adducts moved them to a region of the chromatogram that was relatively free of interference. Third, the molecular ions of the adducts were easily discernible for the alcohol and the acetate (and for standards of the aldehyde), providing confirmation of the molecular weights of the original compounds. Fourth, the diagnostic fragments locating the diene position were unmistakable, with one, the fragment at m/z 208, being the base peak in the spectrum. Fifth, the adducts were more reliably detected at lower concentration than the parent compounds because the adduct fragmentations were concentrated into a few diagnostic ions. Furthermore, because these ions were of distinctive and high molecular weights, they could be easily discerned from background interference. Overall, this method may be even more useful than the isobutane chemical ion-

ization method of determining diene positions (Doolittle et al., 1985) because the latter method gives diagnostic fragments of variable size and does not work well with acetates.

Previous field studies suggested that *Hemileuca* spp. may use both differing daily flight periods and differing pheromone chemistries to achieve reproductive isolation (Collins and Tuskes, 1979; Tuskes et al., 1996). Differences in sex pheromone chemistry have been postulated based on field observations of the relative attractiveness of different populations and species. However, to date, the sex pheromone of only a single species of North American saturniid moth has been reported (*E*6,*Z*11–16 : Ald), that of *Antherea polyphemus* (Cramer), (Kochansky et al., 1975), despite the biology of a number of species being quite well known. The only previous indication of the utilization of 10,12-hexadecadiene compounds as sex pheromone components in the Saturniidae is the observation that males of *Amphion nessus* (Cramer) were attracted to a female *Anisota virginiensis* (Drury) (Willaims, 1985). Bombykal, *E*10,*Z*12–16 : Ald, is known to attract males of *Amphion nessus floridensis* (B.P. Clark) (Landolt et al., 1989), thus suggesting that *A. virginiensis* may be producing this compound.

The major sex pheromone component of *H. eglanterina*, *E*10,*Z*12–16 : Ac, is the major component of the sex pheromone of *Rondotia menciana* Moore (Bombycidae) (Dai et al., 1987, 1988a) and, in combination with the *EE* isomer, has been used for mass-trapping (Dai et al., 1988b). Thus, our study is only the second time this compound has been identified as a moth sex pheromone and the first instance as a sex pheromone for the family Saturniidae. There is evidence that isomers of 10,12–16 : Ac are components of sex pheromones for moths in the family Sphingidae, with *Z*10,*E*12–16 : Ac having been identified as an attractant and eliciting antennal responses from males of *Pachysphinx modesta* (Harris) and *Smerinthus cerisyi* Kirby, and evidence, albeit scant, suggesting that *E*10,*Z*12–16 : Ac may be part of the sex pheromone of *Paonis exceactus* (J. E. Smith) (Reed et al., 1987). Furthermore, extracts of the pyralid moth *Diaphania hyalinata* (L.) are reported to contain trace amounts of *E*10,*E*12–16 : Ac, but it does not appear to be important for attraction (Raina et al., 1986). Our ongoing studies indicate that *E*10,*Z*12–16 : Ac may be widespread as a pheromone component in the genus *Hemileuca* (McElfresh and Millar, personal observation).

This is the first report of *E*10,*Z*12–16 : OH, or bombykol, as a sex pheromone component for a saturniid moth. Since its original isolation from *Bombyx mori* (L.) (Butenandt et al., 1959), bombykol has been identified from the congeneric *B. mandarina* Moore (Kuwahara and My-Yen, 1977) and from the pyralid moth *Notarcha derogata* (F.) (Himeno and Honda, 1992). However, the role of bombykol in the latter species is unclear because neither bombykol nor the *E*10,*E*12–16 : OH isomer were necessary for attraction; aldehydes were the major attractive constituents of the pheromone blend. *E*10,*E*12–16 : OH has now

been identified in *B. mori* female extracts, although its importance to the insect has not been established (Kasang and Schneider, 1978), and in *B. mandarina*, where it did not appear to affect attraction of male moths (Kuwahara et al., 1984). In fact, in all species in which bombykol has been found to date, including *H. eglanterina*, the *E*10,*E*12–16 : OH isomer has also been found, but there is not yet any mention of the other two stereoisomers, *Z*10,*E*12–16 : OH and *Z*10,*Z*12–16 : OH, in the sex pheromone literature (Arn et al., 1995).

In contrast to *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : OH, *E*10,*Z*12–16 : Ald (bombykal) is a relatively common lepidopteran pheromone component, having been reported from moths in several families. It was first identified from female extracts of *B. mori* (Kasang et al., 1978). Soon afterwards it was identified as the major component for the sphinx moth, *Manduca sexta* (Johansson) (Starratt et al., 1979). A number of other sphingid species also have been attracted to lures containing *E*10,*Z*12–16 : Ald, including *Sphinx drupiferarum* (J. E. Smith) and *Hyles gallii* (Rottenburg) (Reed et al., 1987), and *Amphion floridensis* (= *Amphion nessus floridensis* B. P. Clark) (Landolt et al., 1989). Two genera of pyralid moths possess this compound, *Notarcha* and *Diaphania*. In *Notarcha*, both *N. basipunctalis* (Brem.) and *N. derogata* use bombykal as their major pheromone component, but the former also possesses significant amounts of the *E*10,*E*12–16 : Ald isomer, which appears to act as an isolating mechanism between the two species (Honda et al., 1994). For *Diaphania nitidalis* (Stoll) and *D. hyalinata* (L.), bombykal was present in small amounts in female extracts, and in wind-tunnel tests the compound was necessary to duplicate the entire range of male behaviors involved in attraction [*D. hyalinata* (Raina et al., 1986) and *D. nitidalis* (Klun et al., 1986)]. *D. hyalinata* also possessed significant amounts of the *E*10,*E*12–16 : Ald isomer and traces of the *Z*10,*Z*12–16 : Ald, both of which appeared to be important in wind-tunnel studies (Raina et al., 1986). Furthermore, it has been suggested that *E*10,*Z*12–16 : Ald is important in reproductive isolation of two noctuid species, *Earias insulana* (Boisduval) and *E. vittella* (F.), both of which use *E*10,*E*12–16 : Ald as their major pheromone component (Cork et al., 1988). Addition of *E*10,*Z*12–16 : Ald to a multicomponent blend increases attraction of *E. insulana* and decreases attraction of *E. vittella*. In addition to those mentioned above, moth species in the families Schreckensteiniidae and Gracillariidae are known to be attracted to isomers of 10,12–16 : Ald (Arn et al., 1995).

Conducting field trials for optimization of the *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald ratios concurrently resulted in the interesting observation that the ratio of *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald appeared to be more critical than the ratios of either one to the major component, *E*10,*Z*12–16 : Ac. A shift of 10-fold or less from the optimum ratio of the two minor components resulted in significant reductions in trap catches (Figure 3 and 4). In contrast, when the dose of the two minor components was varied as a unit with the

ratio of *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald fixed at 10 : 1, and the amount of *E*10,*Z*12–16 : Ac was held fixed, there was no statistical difference between ratios tested over a range in excess of 100 fold (Figure 6).

In the final trial of a reconstructed blend versus blends with single deletions, only deletion of the three major components in the blend caused a statistically significant reduction in trap catches (Figure 7). However, the limited number of replicates and the variability in catch may have obscured subtle effects because single deletions of 16 : Ac, *Z*11–16 : Ac, *E*11–16 : Ac, and *E*10,*E*12–16 : Ac did appear to lower trap catches by as much as twofold. Thus, we hesitate to exclude the possibility that one or more of these minor components may indeed contribute to the efficacy of the blend. More sensitive tests, such as detailed analysis of male flight behaviors in a wind tunnel, and more detailed examination of the behavior exhibited by the responding males, as was employed in pheromone identifications for *Diaphania nitidalis* and *D. hyalinata*, for example (Klun et al., 1986; Raina et al., 1986), may reveal a role for these components.

The optimum blend of synthetic pheromone on a rubber septum was 100 : 10 : 1 *E*10,*Z*12–16 : Ac to *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald (Figure 6), which differed somewhat from the mean ratio found in single female extracts (100 : 48 : 1.1, Table 1). The increased quantity of *E*10,*Z*12–16 : OH in the gland extracts may reflect that the alcohol is a potential precursor of both the corresponding acetate and the aldehyde. Furthermore, the mean gland content of *E*10,*Z*12–16 : OH was influenced by a single extract that possessed 2.5 times as much *E*10,*Z*12–16 : OH as *E*10,*Z*12–16 : Ac, further suggesting that *E*10,*Z*12–16 : OH may be a precursor or storage form of the two other pheromone components. Removal of this extract from the calculations reduced the mean content of *E*10,*Z*12–16 : OH for single female extracts from 48% to 40% of the *E*10,*Z*12–16 : Ac and halved the range from 6–260% to 6–129% (Table 1). The female aeration had somewhat less *E*10,*Z*12–16 : OH (30% of the amount of *E*10,*Z*12–16 : Ac), but was well within the range found with the single female extracts (Table 1). Much of this variation may be a reflection of the physiological state of the moth (e.g., when the gland extract was made in relation to when the moth began calling).

In summary, the female-produced sex attractant pheromone blend for *H. eglanterina* consists of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald. Several other compounds found in the extracts elicited antennal responses but did not appear to be important in field trials. These compounds are possible precursors or by-products and may elicit antennal responses as a result of their structural similarity to pheromone components. It is possible that these compounds are important to other populations of this moth or to other species. Further studies of the sex pheromones of other members of the genus and of intraspecific differences in the pheromones of geographically isolated populations are in progress.

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SEX PHEROMONE OF NUTTALL'S SHEEP MOTH, *Hemileuca nuttalli*, FROM THE EASTERN SIERRA NEVADA MOUNTAINS OF CALIFORNIA

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Abstract—The sex attractant pheromone blend of *Hemileuca nuttalli* (Lepidoptera: Saturniidae) from the eastern slope of the Sierra Nevada Mountains in California was determined to be a combination of *E*10,*Z*12-hexadeca-10,12-dien-1-yl acetate (*E*10,*Z*12–16 : Ac) and *E*10,*E*12-hexadeca-10,12-dien-1-yl acetate (*E*10,*E*12–16 : Ac). Ratios of the compounds in extracts of female pheromone glands varied around a mean of 100 : 48, although the ratio was not critical in field trials. Blends of synthetic *E*10,*Z*12–16 : Ac and *E*10,*E*12–16 : Ac in 100 : 50, 50 : 100, and 100 : 100 ratios attracted equal numbers of male moths. Field trials also indicated that *E*10,*Z*12–hexadeca-10,12-dien-1-ol (*E*10,*Z*12–16 : OH) and *E*10,*Z*12-hexadeca-10,12-dienal (*E*10, *Z*12–16 : Ald) were antagonistic at rates of 3.3% and 10%, respectively, of the *E*10,*Z*12–16 : Ac, despite being found in female moth extracts. *E*10,*E*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald all appear to have roles in maintenance of reproductive isolation between *H. nuttalli* and *H. eglanterina*.

Key Words—*Hemileuca nuttalli*, *Hemileuca eglanterina*, *E*10,*Z*12-hexadecadienyl acetate, *E*10,*E*12-hexadecadienyl acetate, *E*10,*Z*12-hexadecadienol, *E*10,*Z*12-hexadecadienal, sex pheromone.

INTRODUCTION

Nuttall's sheep moth, *Hemileuca nuttalli* (Strecker) (Lepidoptera: Saturniidae) is a medium-sized, brightly colored, day-flying silk moth found in the Great Basin sage plant community of the western United States. The life history and distribu-

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tion of this moth, as well as most of the other 18 described species in the genus *Hemileuca*, are well known (Tuskes, 1984; Tuskes et al., 1996). The eggs are deposited in rings around twigs of the host plants, antelope bush [*Purshia tridentata* (Pursh)] and snowberry (*Symphoricarpos* species Duhamel de Monceau) in the late summer to early fall. The eggs hatch the following spring, and for the first three instars the larvae are gregarious. After the third instar, the larvae disperse and forage individually. Larval development is completed in one season, after which the larvae construct loose cocoons and pupate in leaf litter. Pupae can remain in diapause for one to several years, and the life-cycle can require two to four years or more to complete. The adults do not feed, and most individuals live less than one week. The flight season is typically from mid-August to late September in the eastern Sierra Nevada mountains. Males fly in the afternoon with peak flight activity extending from 13 : 30 to 16 : 30 hr (Collins and Tuskes, 1979). The geographic range of the moth includes the western states and southern British Columbia in Canada (Tuskes et al., 1996).

The Great Basin sage plant community is the native habitat for not only *H. nuttalli* but also the sagebrush sheep moth, *H. hera* (Harris), and the common sheep moth, *H. eglanterina* (Boisduval). *H. hera* can be distinguished easily from *H. nuttalli* based upon adult wing color and larval host associations. However, adults and larvae of *H. eglanterina* closely resemble those of *H. nuttalli*, and both species often utilize the same host plants in areas where the species coexist. The similarity between the two species initially caused some confusion as to their taxonomic status until Ferguson (1971) clearly separated them based upon sound morphological characters and assigned specific ranks to each. Subsequently, the biology and distribution of all three species have been described in detail (Tuskes et al., 1996).

Further evidence that *H. nuttalli* and *H. eglanterina* are distinct was obtained from observations of their reproductive biology. Field and laboratory matings between females of *H. eglanterina* and males of *H. nuttalli* usually resulted in infertile eggs (Collins and Tuskes, 1979); in the single recorded instance that fertile eggs were obtained, the neonate larvae refused to feed (Collins and Tuskes, 1979). Despite this, a putative hybrid specimen was reared from a field-collected larva, indicating that viable crosses may occur (Collins and Tuskes, 1979; Tuskes et al. 1996).

Reproductive isolation between the two species in the area of Monitor Pass, Alpine County, California, was demonstrated to be due in part to allochrony, with *H. eglanterina* flying in the morning hours and *H. nuttalli* in the afternoon (Collins and Tuskes, 1979). However, there was considerable overlap in flight periods, and unmated *H. eglanterina* females were observed to call well beyond the morning hours. Two observations suggested that the two species had different pheromone chemistry. First, males of *H. nuttalli* responded to calling *H. eglanterina* females, but not the reverse. Second, males of *H. nuttalli*, although

attracted by *H. eglanterina* females, did not exhibit the hovering flight pattern they typically show when in close proximity to a conspecific female (Collins and Tuskes, 1979). The objectives of this study were: (1) to identify the sex pheromone of *H. nuttalli*, and (2) to compare it to that of *H. eglanterina*, in an effort to determine the role of pheromonal mechanisms in maintaining reproductive isolation between these sympatric congeners.

METHODS AND MATERIALS

Insects. Insects used in this study were obtained from the vicinity of Conway Summit, ca. 16 km south of Bridgeport, Mono County, California. Field-collected males were obtained from traps baited with synthetic pheromone lures in late August to early September. Males were placed in labeled glassine envelopes, then in sealed plastic bags with a damp towel and held in an ice chest or refrigerator (4–15°C) until needed for electroantennographic studies. Late-instar larvae were collected in June and July either on or near the larval host, antelope bush [*Purshia tridentata* (Pursh)]. Larvae were reared on bouquets of cut *P. tridentata* or mountain mahogany (*Cercocarpus betuloides* Nuttall) in screen-covered 40-liter glass aquaria. Mature, wandering larvae were placed in plastic boxes containing peat as a pupation substrate and allowed to pupate. Pupae were kept outdoors in shaded, screen cages to allow natural coordination of emergence. *H. nuttalli* requires two or more years to complete development; many of the late-instar larvae collected in 1994 and 1995 did not emerge until 1997. Emerging adults were collected daily and were either used immediately, placed in glassine envelopes (males), or held in 6- × 8-cm screen cages (females) inside sealed plastic bags with a damp towel at 4–10°C for longer term storage (one to two weeks). Cooling was necessary due to the short life-span (less than one week under normal conditions) of the nonfeeding adults. Unmated females generally ceased calling and began depositing unfertilized eggs within three days of eclosion at 22°C. Females were used for preparation of pheromone extracts, while males were used for electroantennographic studies.

Extraction of Sex Pheromone Glands. Individual female sex pheromone glands were removed from virgin calling females of 1–4 days of age. The gland was extruded by applying gentle pressure to the tip of the abdomen to force eversion of the ovipositor, then it and the terminal abdominal segments were excised using iris scissors into a 0.25-ml conical glass vial insert containing approximately 30 μl pentane. The glands were extracted 20 min, and then the solvent was transferred to a clean insert, with two rinses of 1 drop of pentane. Extracts were concentrated by passive evaporation as needed prior to analysis. If not used immediately, extracts were stored at −20°C.

Gas Chromatography. Gas chromatography (GC) analyses were conducted

in splitless mode with Hewlett-Packard 5890A or 5890 Series II gas chromatographs (H-P, Palo Alto, California), by using helium as a carrier gas. Columns and programs used to analyze composite extracts from multiple females included: HP Ultra-1 (30 m × 0.25 mm ID, 0.17-μm film, 100°C for 1 min, 10°C/min to 275°C, hold for 45 min), DB-225 (30 m × 0.26 mm ID, 0.25-μm film, J&W Scientific, Folsom CA; 100°C for 1 min, 10°C/min to 220°C, hold for 45 min), DB-5 (30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, 10°C/min to 275°C, hold for 45 min), and DB-Wax (30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, 10°C/min to 250°C, hold for 45 min). Extracts from individual females were analyzed on a coupled GC-electroantennogram detector (GC/EAD) system, described below.

Coupled Gas Chromatography-Electroantennography (GC-EAD). Aliquots of female extracts and synthetic standards were analyzed by coupled GC-EAD using an H-P 5890 series II GC, with a DB-5 column (30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, 10 or 15°C/min to 275°C, hold for 45 min). The column effluent was split equally with a fused silica press-fit Y-connector (J&W Scientific) into two branches of 0.25-mm-ID, uncoated, fused-silica tubing, with one branch going to the GC flame ionization detector (FID), and the other being directed through a heated transfer line (275°C) to a 1-cm-diam. glass stimulus delivery tube. The capillary effluent was diluted with humidified air (flow of 400 ml/min) and passed over the male moth antenna preparation (see below). The moth antenna was suspended between glass capillary electrodes filled with insect physiological saline (modified Locke's saline, 7.5 g NaCl, 0.21 $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1 liter distilled water) (Humason, 1972), with electrical contact to a custom-built multistage amplifier made with AgCl-coated silver wires. The amplifier and FID outputs were simultaneously recorded on a matched pair of H-P 3394 recording integrators.

Both laboratory-reared and field-collected male moths were used in EAD studies. Male antennae were removed using fine forceps to firmly hold the scape and pull the antenna free of the head, without crushing the scape. The terminal rami and end of the antenna were removed so that the tip of the antenna could be placed into the saline-filled recording electrode. The antenna was positioned on the amplifier mount at the end of the delivery tube such that the plane of the antennal branches was perpendicular to the airflow from the stimulus delivery tube. A single antenna preparation could be used for several hours with periodic changes of saline.

Coupled Gas Chromatography–Mass Spectrometry. Electron impact (EI) mass spectra (70 eV) were taken with an H-P 6890 gas chromatograph interfaced to an H-P 5973 mass selective detector. A 30-m × 0.25-mm-ID HP5-MS column was used in splitless mode, programmed from 50°C at 1 min and 10°/min to 250°, and held for 20 min, injector 275°, transfer line 280°C. Compounds in the insect extract were identified by comparison of retention times and mass

spectra with those of synthetic standards (see below). The sample analyzed was an aliquot of a composite of five female extracts.

Approximately two female equivalents of a composite extract of five females obtained in July 1997 were concentrated under a stream of N_2 to remove pentane, then taken up in 20 μl CH_2Cl_2 and treated with 2.5 μl of a solution of the derivatizing agent 4-methyl-1,2,4-triazoline-3,5-dione (MTAD, 1.2 mg/ml. Aldrich Chemical, Milwaukee, Wisconsin) (Young et al., 1990; McElfresh and Millar, 1999). The resulting faint pink solution was concentrated to <5 μl, and a 1-μl aliquot was analyzed by GC-MS, using an injector temperature of 300°C, and a temperature program of 100°C at 0 min, 15°C min to 300°C, hold for 20 min, and a solvent delay time of 8 min. Identifications were confirmed by analyses of adducts prepared in identical fashion from synthetic pheromone standards.

Synthesis of Pheromone Components. Standards used in chromatographic work and for making lures for field trials were synthesized and purified as described in McElfresh and Millar (1998). GC standards of all four 10,12–16 : Ac isomers were also obtained from Darwin Reed, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada.

Field Trials. Field trials were conducted in late August to early September of 1994, 1995, and 1996 at a site 16 km south of Bridgeport, California, where larvae had been obtained during June and July of 1994 and 1995 (see above). Cylindrical funnel traps (15 cm OD × 25 cm high) fabricated from metal screen (described in detail in McElfresh and Millar, 1999), were used. Lures consisted of 11 mm red rubber septa (Wheaton Scientific, Millville, New Jersey), individually labeled with a treatment number, and loaded with heptane solutions (100 μl) of test compounds and blends, plus 2 drops of 10 mg/ml butylated hydroxytoluene (BHT) in heptane as an antioxidant. Once the treatment solutions had soaked into the septa, the lures were impaled on fishhooks and stored in 20-ml glass vials for transport. Lures were kept in a freezer or an ice-chest when not in use. No one set of lures was used for more than five days.

A single trial consisted of three replicate blocks located a minimum of 0.5 km apart adjacent to roads in the area. The number of traps used was three times the number of treatments or rates tested plus a blank control (e.g., 24 traps for a field trial of eight treatments including the control). Two to three trap counts were made at one-day intervals, with the traps being cleaned out and rerandomized after each reading. Traps were placed ~10 m apart in a straight line in areas that supported high densities of the moth. Trap counts were generally made in the early to late afternoon.

Statistics. For the statistical analysis of field trials, elimination of the effect of day was accomplished by pooling the day counts for each treatment in a given block. This sum was then transformed ($\sqrt{x}$ + 0.5) prior to conducting a two-way ANOVA, followed by linear and quadratic contrasts. If significant effects

were detected, then the Student-Neuman-Keuls (SNK) test was used to separate means (SAS Institute, 1996).

RESULTS

Analysis of Female Extracts. Analysis of female pheromone gland extracts by coupled GC-EAD demonstrated that male moth antennae responded to several components in the extracts (Figure 1). These compounds, and several others present in the extracts, were identified by GC and GC-MS (Table 1). The mass spectrum of the component eliciting the strongest antennal response (Figure 1, compound **7**; Table 1) was consistent with that of a conjugated 16-carbon diene acetate, with a comparatively strong molecular ion (*m/z* 280, 20% of base peak, possible molecular formula $C_{18}H_{32}O_2$), and a small but distinct fragment at *m/z* 220 (2%, loss of acetic acid). Comparison with a database spectrum of *E*10,*Z*12–16 : Ac (NIST/EPA/NIH Mass Spectral Database, 1992) resulted in a close match. The position of the conjugated double bonds was confirmed by

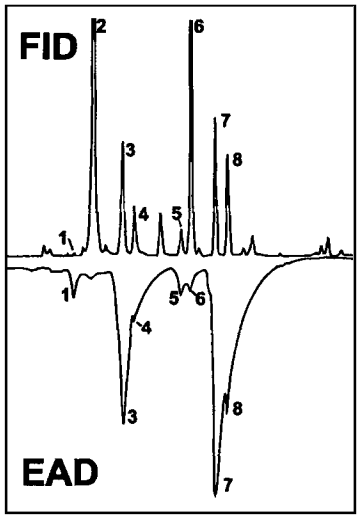

FIG. 1. Coupled gas chromatogram–electroantennogram of male *H. nuttalli* antenna stimulated by a female extract. Column used was DB-5 (30 m × 0.32 mm ID, 0.25-μm film, temperature program 100°C for 1 min, 10°C/min to 275°C, hold for 45 min). Identifications of peaks: (1) *E*10,*Z*12–16 : Ald, (2) 16 : OH, (3) *E*10,*Z*12–16 : OH, (4) *E*10,*E*12–16 : OH, (5) *E* and *Z*11–16 : Ac, (6) 16 : Ac, (7) *E*10,*Z*12–16 : Ac, and (8) *E*10,*E*12–16 : Ac.

TABLE 1. COMPOUNDS, METHODS OF IDENTIFICATION, AND AMOUNTS OF COMPOUNDS RELATIVE TO *E*10,*Z*12–16 : Ac IN EXTRACTS OF FEMALE *Hemileuca nuttalli*

Compound	Identification methods used[a]		Amount in single female extracts relative to *E*10,*Z*12–16 : Ac[b]			
	GC	GC-MS	Mean	SD	Range	% detected[c]
*E*10,*Z*12–16 : Ac	X	X	100			100
*E*10,*E*12–16 : Ac	X	X	47.6	32.6	5.9–154	100
16 : Ac	X	X	160	127	18.3–529	100
E/*Z*11–16 : Ac	X		17.2	7.7	5.5–33.4	94
Z10–16 : Ac	X		2.5	2.9	0.2–9.8	29
16 : OH	X	X	261	598	4.3–2745	87
*E*10,*Z*12–16 : OH	X	X	38.4	77.6	5.7–368	65
*E*10,*E*12–16 : OH	X		18.2	46.3	0.2–195	52
*E*10,*Z*12–16 : Ald	X		1.0	0.37	0.3–1.6	39

[a]GC = retention time matches on at least three columns (see materials and methods) with authentic standards. GC-MS = mass spectral match with authentic standards.
[b]Means of 31 female extracts.
[c]Percent of female extracts with detectable amounts.

GC-MS analysis of the Diels-Alder adduct formed with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) (Young et al., 1990; McElfresh and Millar, 1999). The adduct showed a molecular ion at m/z 393 (6%), and diagnostic fragments at m/z 350 (19%) and 208 (100%) from cleavages of the alkyl groups attached to the three and six positions of the six-membered ring of the adduct, which definitively placed the conjugated diene in the parent molecule at the 10,12 positions. Determination of the double-bond stereochemistries was made by comparison of retention times of the insect-produced compounds with those of authentic standards on four GC columns of differing polarity (HP-1, DB5-MS, DB-225, DB-Wax). Only the *E*10,*Z*12–16 : Ac isomer matched the retention time of the insect-produced compound on all four columns. Furthermore, GC-EAD analysis of the isomers of 10,12–16 : Ac indicated that the *E*10,*Z*12–16 : Ac elicited the largest response from male antennae (mean mV ± SD of five males tested: *EZ*, 0.60 ± 0.14; *ZE*, 0.21 ± 0.07; *ZZ*, 0.12 ± 0.03 and *EE*, 0.14 ± 0.02; one-way ANOVA, $F = 26.5$, $df = 19$, $P < 0.0001$).

In addition to the *E*10,*Z*12–16 : Ac isomer, the insect extracts contained significant amounts of *E*10,*E*12–16 : Ac (Figure 1, compound **8**; Table 1). The mass spectrum of this isomer was virtually indistinguishable from that of *E*10,*Z*12–16 : Ac, but it eluted later on all four GC columns used (see McElfresh and Millar, 1999 for retention time tabulations of all four isomers on four columns). GC-EAD responses to *E*10,*E*12–16 : Ac were much smaller than those to the *E*10,*Z*12–16 : Ac, even when equal amounts of each isomer were presented to the antenna (see above). The size of responses to *E*10,*E*12–16 : Ac

TABLE 2. RELATIVE SIZES AND FREQUENCY OF ELECTROANTENNOGRAPHIC RESPONSES BY MALE ANTENNAE[a] OF *Hemileuca nuttalli* FROM EASTERN SIERRA NEVADA MOUNTAINS TO COMPOUND IN FEMALE EXTRACTS[b]

Compound	Response relative to *E*10,*Z*12–16 : Ac female extracts			
	Mean	SD	Range	% detected[c]
*E*10,*Z*12–16 : Ac	100			100
*E*10,*E*12–16 : Ac	50.5	9.0	35–69	91
E/*Z*11–16 : Ac	10.9	8.9	3–46	100
16 : Ac	8.9	7.2	3–35	100
*E*10,*Z*12–16 : OH	22.4	17.3	4–70	95
*E*10,*E*12–16 : OH	13.5	5.3	8–21	18
*E*10,*Z*12–6 : Ald	5.9	4.1	1–15	86

[a]Total of 5 males tested.
[b]Total of 22 female extracts tested.
[c]Percent of female extracts which elicited responses by male antennae.

in antennograms of female extracts may be misleading due to the overlap with the *E*10,*Z*12–16 : Ac response (Figure 1), but responses to *E*10,*E*12–16 : Ac in extracts were consistent (Table 2). Final confirmation of *E*10,*Z*12–16 : Ac and *E*10,*E*12–16 : Ac as the correct structures was obtained from the results of field trials (see below).

The compound eliciting the second largest GC-EAD response (Figure 1, compound **3**), was identified as *E*10,*Z*12–16 : OH. Specifically, the EI mass spectrum showed a molecular ion at m/z 238 (19%), corresponding to a possible molecular formula of $C_{16}H_{30}O$, and the spectrum matched that of an authentic standard. Furthermore, the MTAD adduct exhibited a molecular ion at m/z 351 (8%), and diagnostic fragments at m/z 208 (100%) and 308 (32%), fixing the diene position. The stereochemistry of the double bonds was determined through retention time comparisons with standards of the four isomers. The retention times of the insect-produced compound on the four GC columns matched only those of the *E*10,*Z*12–16 : OH isomer. Lesser amounts of the *E*10,*E*12–16 : OH isomer were also tentatively identified in extracts by retention time matches with a standard on four GC columns, and by GC-EAD (Figure 1, compound **4**).

In addition to these four compounds, several other compounds in the extracts elicited responses from male antennae in GC-EAD runs or were present in significant amounts (Figure 1, Tables 1 and 2). These compounds included the saturated compounds 16 : OH and 16 : Ac (Figure 1, compounds **2** and **6**), readily identified from comparison of mass spectra and retention times with those of standards. The dienal *E*10,*Z*12–16 : Ald **1** (Fig. 1) was tentatively identified by retention time comparisons with standards of all four 10,12–16 : Ald isomers on the four GC columns,

but concentrations in extracts were too low to obtain a mass spectrum. In addition, *E*- and *Z*11–16 : Ac, and *Z*10–16 : Ac were tentatively identified by comparison of retention times with standards on four GC columns. In particular, the monoene acetates were identified by first determining the retention times on DB-Wax of the *E* and *Z* isomers of hexadecenyl acetates in which the double bond position ranged from carbon 5 to 13. Comparison of retention times narrowed the possibilities down to *Z*10–16 : Ac and *E*11–16 : Ac for the first peak and *Z*11–16 : Ac, *E*12–16 : Ac, and *E*13–16 : Ac for the second peak. For the second peak, only the *Z*11–16 : Ac standard matched retention times on the other three columns.

Identification of the other two monoene acetates was more complex. Using *Z*11–16 : Ac and 16 : Ac as internal references, comparison of retention times on HP-1, DB-5, and DB-Wax columns indicated that *Z*10–16 : Ac was present. In particular, the relative areas of the *Z*11–16 : Ac and *Z*10–16 : Ac peaks in a gland extract were different on DB-Wax (2.8 : 1) and DB-5 (5.5 : 1). This discrepancy in ratios can be explained by the fact that on DB-Wax, *Z*10– and *E*11–16 : Ac coelute, whereas on DB-5, *E*11– and *Z*11–16 : Ac coelute. Thus, on DB-Wax, the peak at the retention time of *Z*10–16 : Ac is actually a composite with *E*11–16 : Ac, and the *Z*11–16 : Ac is due to the single compound, while on DB-5, the situation is reversed, with the *Z*10–16 : Ac peak arising from a single component, and the *Z*11–16 : Ac peak now being a composite with *E*11–16 : Ac. Thus, all three isomers (*Z*11–16 : Ac, *Z*10–16 : Ac, and *E*11–16 : Ac), were tentatively determined to be in female extracts.

Overall, the mean ratio of *E*10,*Z*12–16 : Ac to *E*10,*E*12–16 : Ac in 31 female extracts was 100 : 48 (Table 1). The remaining compounds identified, *E*10,*Z*12–16 : OH, *E*10,*E*12–16 : OH, *E*10,*Z*12–16 : Ald, *E*/*Z*–16 : Ac, *Z*10–16 : Ac, 16 : Ac, and 16 : OH, were present in a mean ratio of 38 : 18 : 1 : 17 : 3 : 160 : 262 (expressed as a percentage of the *E*10,*Z*12–16 : Ac, Table 1). These ratios, and the relative responses of male antennae in GC-EAD studies (Figure 1) were used to guide formulation of blends for field trials. *E*10,*Z*12–16 : Ac, which elicited the strongest response from antennae, was considered the major component of the pheromone when optimizing the ratio of the blends in field trials, with the other components expressed as a percentage of this compound.

Field Trials. While conducting field trials of attraction of male *H. eglanterina* in 1994 (McElfresh and Millar, 1999), we determined that blends of both *E*10,*Z*12–16 : Ac and *E*10,*E*12–16 : Ac were necessary for attraction of male *H. nuttalli* (Figure 2). Thus, a blend of 100 : 10 : 1 μg *E*10,*Z*12–16 : Ac to *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald, the optimally attractive *H. eglanterina* pheromone blend, was spiked with two rates of *E*10,*E*12–16 : Ac (100 and 10 μg/bait). At the lower rates (0 and 10 μg/bait added), significantly more *H. eglanterina* were trapped than with baits containing 100 μg *E*10,*E*12–16 : Ac (Figure 2). Conversely, fewer *H. nuttalli* were attracted by the baits containing

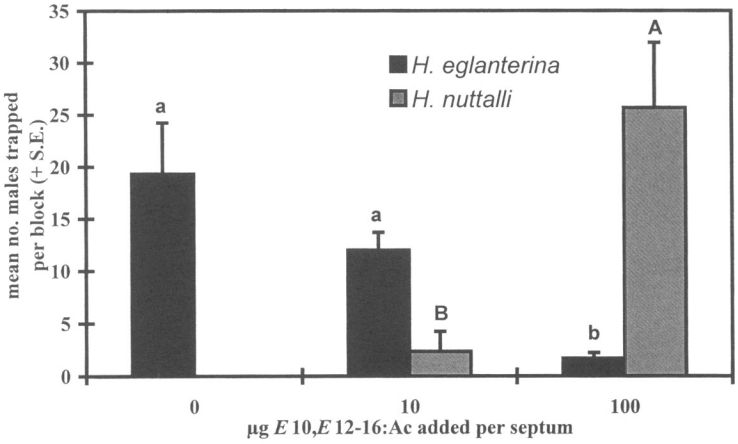

FIG. 2. Trap catches of male *Hemileuca nuttalli* (Strecker) and *H. eglanterina* (Boisduval) in field trials in the vicinity of Conway Summit, Mono County, California. The *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald rates were held constant at 100 : 10 : 1 µg/septum, respectively, while the amount of *E*10,*E*12–16 : Ac added was varied. The trial was conducted from August 20 to 22, 1994. Total number of moths trapped was 84 for *H. nuttalli* and 99 for *H. eglanterina*. Treatments within species with different letters are significantly different (SNK, $\alpha = 0.5$). *H. nuttalli* for *E*10,*E*12–16 : Ac effect; $F = 9915.1$, $df = 1,2$, $P = 0.0001$; no contrasts possible. *H. eglanterina* for *E*10,*E*12–16 : Ac effect, $F = 15.9$, $df = 2, 4$, $P = 0.0125$; linear contrast $F = 30.0$, $df = 1$, $P = 0.0054$, quadratic contrast $F = 1.76$, $df = 1$, $P = 0.2554$.

low rates of *E*10,*E*12–16 : Ac than to the high rate bait (Figure 2), indicating the importance of the *E*10,*E*12–16 : Ac isomer to this species.

Our first formal field trial for *H. nuttalli* reexamined the ratios of these two isomers, without *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald. The blends tested consisted of the purest materials available at the time [99% *E*10,*E*12–16 : Ac (*EE*) to 1% *E*10,*Z*12–16 : Ac (*EZ*) and 97% *EZ* to 3% *EE*]. The 99% *EE* was unattractive, and the 97 : 3 *EZ–EE* was significantly less attractive than the 100 : 100 and 100 : 50 *EZ–EE* blends, which were the most attractive but not significantly different from each other (Figure 3). Based upon this information, the basic blend used for subsequent field trials was 100 : 50, *E*10,*Z*12–16 : Ac to *E*10,*E*12–16 : Ac, close to the 100 : 47.6 blend found in pheromone gland extracts (Table 2).

A second field trial tested the role of *E*10,*Z*12–16 : OH, the compound in extracts that elicited the second largest response from male antennae, as a pheromone component. Despite the considerable amount of this material in extracts (Table 2), even relatively small amounts of this compound significantly decreased attraction to traps (Figure 4), and larger doses of *E*10,*Z*12–16 : OH

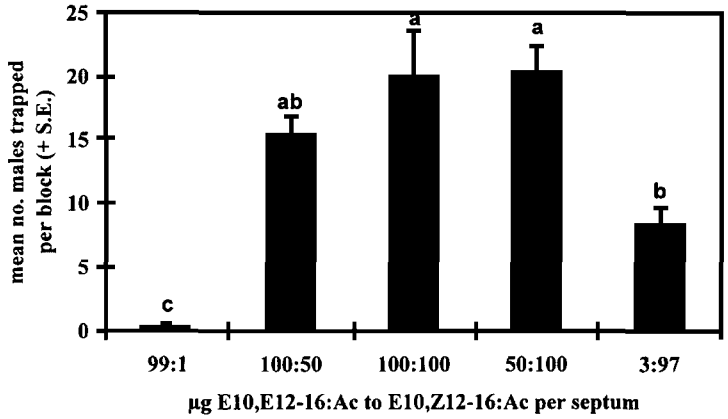

FIG. 3. Trap catches of male *Hemileuca nuttalli* in field trials, testing the ratio of *E*10,Z12–16 : Ac (*EZ*) to *E*10,*E*12–16 : Ac (*EE*). The trial was conducted from August 12 to 14, 1995. Total number of moths trapped was 192. Treatments with different letters are significantly different (SNK, $\alpha = 0.5$). Two-way ANOVA for ratio *EZ–EE* effect, $F = 20.7$, $df = 4, 8$, $P = 0.0003$; linear contrast $F = 18.8$, $df = 1$, $P = 0.0025$, quadratic contrast $F = 61.7$, $df = 1$, $P = 0.0001$.

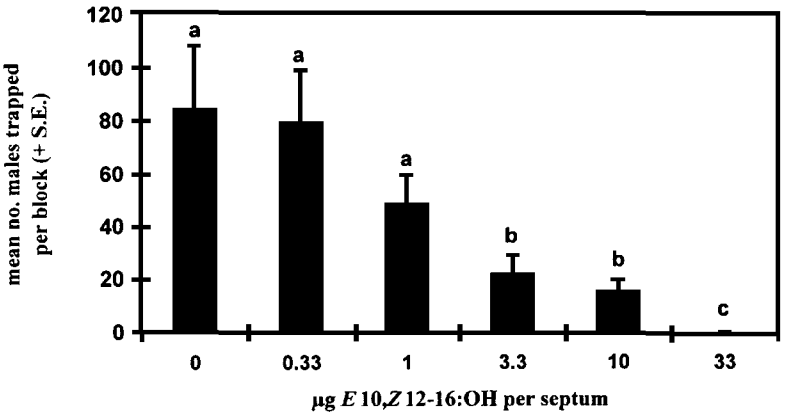

FIG. 4. Trap catches of male *Hemileuca nuttalli* in field trials testing additions of *E*10,Z12–16 : OH to standard baits with *E*10,Z12–16 : Ac and *E*10,*E*12–16 : Ac held constant at 100 and 50 µg/septum, respectively. The trial was conducted on August 27 and 28, 1995. Total number of moths trapped was 745. Treatments with different letters are significantly different (SNK, $\alpha = 0.5$). Two-way ANOVA *E*10,Z12–16 : OH effect, $F = 22.4$, $df = 5, 10$, $P = 0.0001$; linear contrast $F = 107.2$, $df = 1$, $P = 0.0001$, quadratic contrast $F = 2.1$, $df = 1$, $P = 0.1746$.

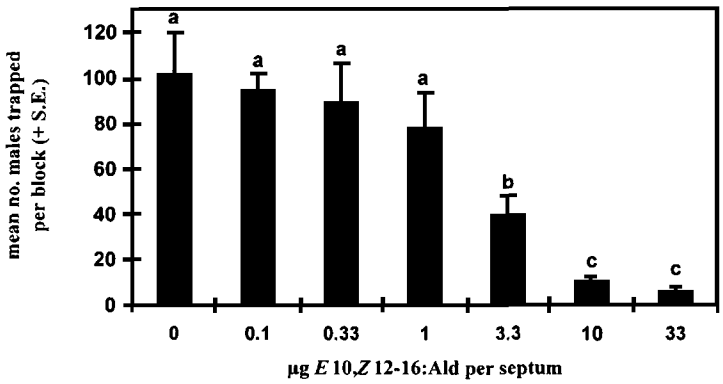

FIG. 5. Trap catches of male *Hemileuca nuttalli* in field trials testing additions of *E*10,*Z*12–16 : Ald to standard baits with *E*10,*Z*12–16 : Ac and *E*10,*E*12–16 : Ac held constant at 100 and 50 µg/septum, respectively. The trial was conducted on August 27 and 28, 1995. Total number of moths trapped was 1241. Treatments with different letters are significantly different (SNK, $\alpha = 0.5$). Two-way ANOVA for *E*10,*Z*12–16 : Ald effect, $F = 24.3$, $df = 6, 12$, $P = 0.0001$; linear contrast $F = 116.3$, $df = 1$, $P = 0.0001$, quadratic contrast $F = 10.3$, $df = 1$, $P = 0.0076$.

completely eliminated trap captures. In an analogous test with two rates (0, 3.3, and 33 µg) of *E*10,*E*12–16 : OH added to the standard two-component bait, no significant differences in attractiveness were seen (mean ± SD no additive, 62.3 ± 38.6 moths; 3.3 µg, 47.3 ± 18.9; 33 µg, 21.3 ± 10.4; two-way ANOVA, *E*10,*E*12–16 : OH rate effect, $F = 2.9$, $df = 2,4$, $P = 0.1694$; 393 moths trapped). However, the high variability and the limited number of replicates (three, counted two times) may have obscured subtle differences between baits.

In a field trial with *E*10,*Z*12–16 : Ald as a minor component of the bait, increasing amounts of this compound also antagonized attraction (Figure 5). Furthermore, attractiveness of baits containing the lower doses of *E*10,*Z*12–16 : Ald did not differ from that of baits without this compound, indicating that it is probably not part of the attractant blend, even though it appears to be present in the insect extracts in trace amounts.

16 : Ac does not appear to be a pheromone component. In a field trial comparing baits containing 100 : 50 µg *E*10,*Z*12–16 : Ac + *E*10,*E*12–16 : Ac, and an additional dose of 16 : Ac in semilog steps (0, 3.3, 10, 33, 100, 333 µg), all baits were equally attractive (two-way ANOVA, 16 : Ac rate effect, $F = 1.4$, $df = 5$, 10, $P = 0.3153$; three blocks counted twice, total of 1264 moths caught). In a further field trial testing addition of the monoene acetates (at rates approximating those found in gland extracts) to the standard 100 : 50 µg *E*10,*Z*12–16 : Ac + *E*10,*E*12–16 : Ac lure, a 16 : Ac treatment (200 µg) was also included. Nei-

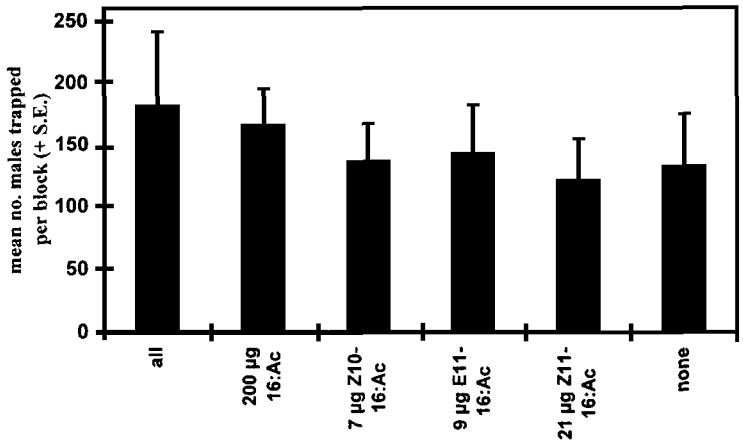

FIG. 6. Trap catches of male *Hemileuca nuttalli* in field trials testing additions of single rates of 16 : Ac, *Z*10–16 : Ac, *Z*11–16 : Ac, and *E*11–16 : Ac alone, and all four together, to standard baits with *E*10,*Z*12–16 : Ac and *E*10,*E*12–16 : Ac held constant at 100 and 50 µg/septum, respectively. Ratios of compounds were similar to those found in female gland extracts. The trial was conducted August 24 and 25, 1995. Total number of moths trapped was 2613. Treatments were not significantly different (two-way ANOVA for additive(s), $F = 1.0$, $df = 5, 10$, $P = 0.4561$).

ther the monoene acetates nor 16 : Ac, singly or in combination, significantly affected the attractiveness of the baits relative to the two-component, 100 : 50 µg *E*10,*Z*12–16 : Ac + *E*10,*E*12–16 : Ac standard lure (Figure 6).

DISCUSSION

Studies of the reproductive biology of *H. eglanterina* and *H. nuttalli* have suggested that diurnal activity patterns are responsible in part for maintaining reproductive isolation between the two species (Collins and Tuskes, 1979; Tuskes et al., 1996). Their close taxonomic relationship, coupled with observations that male *H. nuttalli* are attracted to calling *H. eglanterina* females, suggested similarities between their sex pheromones. However, the pheromones of the two species were not identical, because cross-attraction was unidirectional; *H. eglanterina* males were not attracted to *H. nuttalli* females. The recent identification of the pheromone of *H. eglanterina* (McElfresh and Millar, 1999), coupled with the work reported here, has elucidated the complex role of minor components as synergists and antagonists, enhancing attraction of conspecifics and deterring attraction of congenerics respectively.

Several aspects of the pheromone systems of the sympatric populations

of the two species, including male antenna responses, female extract composition, and male behavioral responses, for the four behaviorally active compounds (*E*10,*Z*12–16 : Ac, *E*10,*E*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald) found in pheromone gland extracts of both species provide some interesting comparisons, as follows. First, the pattern of antennal responses of males of both species to these four compounds was similar, with *E*10,*Z*12–16 : Ac eliciting the strongest responses, followed in decreasing order by *E*10,*E*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald (Table 2 and McElfresh and Millar, 1999). Even the pattern of responses of male antennae from both species to all four 10,12-16 : Ac isomers was similar, in that the *EZ* gave the largest response, followed by *ZE*, *EE*, and *ZZ*, respectively (see above and McElfresh and Millar, 1999). Second, pheromone gland extracts from females of the two species were similar, differing primarily in the amount and range of *E*10,*E*12–16 : Ac (Table 1 and McElfresh and Millar, 1999). While there is considerable overlap in the range of amounts of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald in extracts of the two species, the mean amount of *E*10,*E*12–16 : Ac relative to *E*10,*Z*12–16 : Ac is five times higher in *H. nuttalli* than in *H. eglanterina*, supporting its suspected role as a contributing factor to reproductive isolation. Furthermore, the gland extracts of *H. nuttalli* were similar to those of *H. eglanterina* in the amounts of *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald present, despite the fact that these two components antagonized conspecific male responses in the former species, but were essential for male responses in the latter species (McElfresh and Millar, 1999). Third, the behavioral responses of male moths in field trials clarified the role of pheromone components in reproductive isolation of the two species. *E*10,*Z*12–16 : Ac appears to be the major pheromone component for both species, but *H. nuttalli* males are most strongly attracted to lures containing relatively large amounts of *E*10,*E*12–16 : Ac, although the ratio of the two isomers does not appear to be critical (Figure 3), while *H. eglanterina* responses are antagonized by higher rates of this isomer. Two additional compounds found in female extracts, *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald, were inhibitory to *H. nuttalli* males when added at rates in excess of 3.3 and 10 μg/bait, respectively to the 100 : 50 blend of *E*10,*Z*12–16 : Ac to *E*10,*E*12–16 : Ac (Figure 3 and 4). Conversely, these compounds were essential for attracting male *H. eglanterina*, and the ratio of these two components to each other was critical (McElfresh and Millar, 1999).

The fact that the amount of *E*10,*Z*12–16 : OH found in *H. nuttalli* female gland extracts was highly variable, coupled with the observations that this compound antagonizes the response of male *H. nuttalli*, suggests that this compound may be a pheromone precursor rather than a component of the pheromone blend. As a pheromone precursor, the amount found in gland extracts would be expected to vary between individuals as a function of how much had been converted into other compounds and/or released.

Although moths in the family Saturniidae are known to use sex attractant pheromones, there has been minimal investigation of the chemistry of the compounds involved. To date, sex pheromones of only five species in four genera have been reported (Arn et al., 1995). A few species are reared for silk production, but in general, the family contains few economically important species. However, alternate uses for saturniid species pheromones may arise, because in the United States some saturniid species are close to becoming endangered, in which case sex pheromones may be useful in monitoring populations. Thus, efforts to obtain a more comprehensive knowledge of the breadth and diversity of pheromone chemistry in this moth family are justified.

In summary, the sex pheromone blend for *H. nuttalli* in the eastern Sierra Nevada mountains has been determined to be a combination of *E*10,*Z*12–16 : Ac and *E*10,*E*12–16 : Ac. The ratio of the two components is not critical, with blends ranging from 1 : 2 to 2 : 1 being equally attractive. Two other EAD-active components found in pheromone gland extracts, *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald, antagonize the response, while yet other components of the extract, such as *E*- and *Z*11–16 : Ac, *Z*10–16 : Ac, 16 : OH, and 16 : Ac, have had no discernible effect on male moth behavior. The presence or absence of significant quantities of *E*10,*E*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald in sex pheromone blends appears to be a key factor in the reproductive isolation of the sympatric populations of *H. nuttalli* and *H. eglanterina* that we studied. However, it must be emphasized that the roles of the various pheromone components in other locales, where only one moth species is present, or where other congeneric species are present, is not yet known. Variability in the pheromone blend and in male moth behavior in response to the presence or absence of competition for pheromone channels from sympatric congeners are currently under study.

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HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF SUGARS IN SNAIL-CONDITIONED WATER AND MUCUS FROM *Biomphalaria glabrata*, *Helisoma trivolvis*, AND *Lymnaea elodes*

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Abstract—The presence of sugars, particularly glucose, maltose, and sucrose, in the SCW of *Biomphalaria glabrata*, the two strains of *Helisoma trivolvis*, and *Lymnaea elodes* was variable, although only maltose was quantifiable in two samples of snail conditioned waters from *B. glabrata*. The only sugar detected in mucus was glucose, but it was below the lowest quantifiable amount in the mucus samples of *B. glabrata* and *H. trivolvis*. The mean percentage of glucose in the mucus of *L. elodes* was 0.0040% ± 0.00067%.

Key Words—High-performance thin-layer chromatography, *Biomphalaria glabrata*, *Helisoma trivolvis*, *Lymnaea elodes*, sugars, glucose, maltose, snail conditioned water, mucus.

INTRODUCTION

Snail conditioned water (SCW) is the term used to describe the water containing chemicals released by gastropod mollusks. SCW from pulmonate snails contains chemicals that attract larval trematodes, particularly the miracidial and cercarial stages of numerous digeneans [see review in Haas and Haberl (1997)]. Pulmonate snails, mainly in the families Planorbidae and Lymnaeidae, serve as vec-

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tors of medically and economically important trematodes in the genera *Schistosoma*, *Fasciola*, and *Echinostoma*.

Recent studies in our laboratory have been concerned with substances in SCW that may serve as chemical attractants for larval trematodes. These include high-performance thin-layer chromatographic (HPTLC) analysis of neutral lipids in SCW of *Biomphalaria glabrata* (Chaffee et al., 1996) and *Helisoma trivolvis* (Rivas et al., 1997a) and phospholipids in SCW of these snails (Gennaro et al., 1996). Muller et al. (1999) analyzed neutral lipids and phospholipids in SCW from *Lymnaea elodes*. Steiner et al. (1998) performed HPTLC analysis of amino acids in SCW from *B. glabrata*, *H. trivolvis*, and *L. elodes*, and Rivas et al. (1997b) examined metallic ions in SCW from *B. glabrata* and *H. trivolvis*.

The rationale for the previous studies on lipids, amino acids, and metallic ions in pulmonate snails was clear since all of these compounds have been implicated as chemical attractants for larval trematodes (see Haas and Haberl, 1997). Less information is available on the role of sugars as potential chemoattractants for larval trematodes. Haas and Haberl (1997) have pointed out studies that indicate that sugars may serve as chemoattractants for larval trematodes, and Plorin and Gilbertson (1985) provided experimental evidence to show that, at least under laboratory conditions, glucose can serve as a miracidial attractant for *Schistosoma mansoni*.

This paper reports the results of qualitative and quantitative HPTLC analysis of carbohydrates in the SCW of various pulmonate snails, i.e., *H. trivolvis* (Colorado and Pennsylvania strains), *B. glabrata* (NMRI strain), and *L. elodes* [a strain from northern Indiana described by Sorensen et al. (1997)]. Wilson (1968) detected glucose in the mucus of *L. truncatula*, a snail intermediate host of *Fasciola hepatica*, and speculated on the possible role of mucus as a potential chemoattractant for larval trematodes. Because of Wilson's (1968) report, we extended our carbohydrate analyses to snail mucus.

METHODS AND MATERIALS

Sample Preparation. Stock cultures of *B. glabrata*, the two strains of *H. trivolvis*, and *L. elodes* were maintained as described previously (Frazer et al., 1997) in chemically defined spring water (Ulmer, 1970). To obtain SCW from *B. glabrata* and both strains of *H. trivolvis*, eight snails with shell diameters of 8–15 mm were placed in a 20-ml beaker with 2.0 ml of artificial spring water. To obtain SCW from *L. elodes*, eight snails with shell lengths of 17–27 mm were placed in a 20-ml beaker with 2.0 ml of artificial spring water. Snails were incubated at 28°C for 4 hr, removed with forceps, and rinsed with 90 μl of artificial spring water. Sample and rinse water were transferred with a Pasteur

pipet into a 4-ml vial and evaporated to dryness with a stream of air in a 50–60°C water bath. The residue was reconstituted in 50.0 μl of 70% ethanol. A total of four samples of SCW was used for each strain of *H. trivolvis* and for *B. glabrata* and *L. elodes*.

To obtain mucus, the snail foot of five snails of each taxon was stroked gently with a glass rod as described by Wilson (1968). The pooled mucus was placed on a preweighed piece of aluminum foil and a wet weight was obtained. The weight of sample varied from trial to trial and also with species or strain used. Thus, pooled mucus samples varied from a low of 4 mg in one trial using *H. trivolvis* (PA strain) to a high of 116 mg using *L. elodes* samples in another trial. Sugars were extracted by rinsing the foil with 4.0 ml of 70% ethanol. The extract was transferred to a 4 ml sample vial with a Pasteur pipet, blown to dryness with an air stream in a 50–60°C water bath, and reconstituted in 50.0 μl of 70% ethanol. Two samples of mucus were used for each strain of *H. trivolvis* and *B. glabrata*, and four for *L. elodes*.

HPTLC of Sugars. Qualitative analysis of sugars from SCW was performed on Merck (EM Separations Technology, Gibbstown, New Jersey) 20- × 10-cm HPTLC silica gel 60 CF_{254} plates with 19 lanes and a preadsorbent zone. Plates were impregnated with 0.1 M sodium bisulfite and citrate buffer as described by Anderton et al. (1993). To verify which sugars were present in SCW, 8.00 μl each of 100 ng/μl solutions of glucose, maltose, trehalose, and sucrose (standards purchased from Sigma, St. Louis, Missouri) and 7.00 μl of reconstituted SCW sample from each strain of snail were spotted onto the preadsorbent zone using a Drummond (Broomall, Pennsylvania) 10-μl digital microdispenser. The plate was developed three times with acetonitrile–distilled water (85 : 15) (mobile phase 1) in a paper-lined Camag (Wilmington, North Carolina) twin-trough HPTLC chamber to a distance of 7 cm past the preadsorbent–silica gel interface. The development time per run was 15–20 min. After development, the plate was heated for 2 min with a hair dryer, sprayed with α-naphthol–sulfuric acid detection reagent as described by Anderton et al. (1993), and heated in a 110°C oven for 5 min. Sugar zones appeared purple against a beige background. Zone identification was based on comparison of the R_f values of sample and standard zones.

For quantitative analysis of sugars from SCW, Whatman (Clifron, New Jersey) 20- × 20-cm LK5DF silica gel TLC plates with 19 lanes and a preadsorbent zone were used. Plates were precleaned by development in dichloromethane–methanol (1 : 1) and air-dried under a fume hood. Volumes of 3.00, 6.00, and 8.00 μl of a 100 ng/μl sugar standard and 1.20 μl of a 100 ng/μl sugar standard (0.300–1.20 μg) were spotted on a plate, along with 20.0 μl of reconstituted sample. Plates were developed with ethyl acetate–acetic acid–methanol–distilled water (60 : 15 : 15 : 10) (mobile phase 2) to a distance of 16 cm beyond the layer interface in a Camag twin-trough TLC chamber. Devel-

opment took 2–2.5 hr. Detection of sugar zones was carried out as described above.

For analysis of sugars in mucus, Whatman 20- × 10-cm LHPKDF silica gel 60A HPTLC plates with 19 lanes and a preadsorbent zone and mobile phase 2 were used. Procedures were identical to those used for the quantitative SCW analysis, except plates were allowed to develop to 7 cm past the layer interface, and either 10.0 or 20.0 μl of the reconstituted mucus sample was spotted, depending upon the quantity present in the sample. The development time was approximately 30 min.

Scanning of standard and sample sugar zones was performed using a Camag TLC Scanner II with a tungsten light source set at 515 nm, slit width 4, slit length 5, and scanning rate 4 mm/sec. A calibration curve was generated by the CATS-3 software that related the weights of the standard zones to their peak areas. The weights of sugars in sample zones were interpolated automatically from the calibration curve based on their peak areas. The weight of sugar in SCW was calculated using the following equation: micrograms of sugar in 2.0 ml SCW from eight snails = $(W \times R)/S$, where W is the interpolated weight in micrograms from the calibration curve, R is the reconstitution volume (50.0 μl), and S is the volume of sample spotted (20.0 μl). The weight percent of sugar in mucus was calculated using the following equation: % sugar = $W \times (R/S) \times 1/M \times 100$, where M is the weight of the mucus sample and S is the volume of samples spotted (10.0 or 20.0 μl).

RESULTS

Snails were incubated at 28°C to obtain the maximum release of chemoattractants in SCW. Preliminary studies showed that most strains incubated at 22–24°C released only trace amounts of biochemicals. TLC analyses of sugars in SCW from *H. trivolvis* (PA and CO strains) and *L. elodes* with mobile phase 2 produced variable results. No sugars were detected in four separate trials from the PA strain of *H. trivolvis*. For the CO strain of *H. trivolvis*, maltose (R_f = 0.36) and glucose (R_f = 0.47) were detected in two of four trials. In one of four trials for *L. elodes*, maltose was detected. The concentrations of these detected sugars were below 0.047 mg/ml/snail, which was the lowest amount that could be quantified. However, two of four samples from *B. glabrata* yielded quantifiable concentrations of maltose of 0.13 and 0.073 mg/ml/snail. The other two samples displayed concentrations of maltose below the lowest quantifiable amount. A faint sucrose band (R_f = 0.41) was present in one of the four analyses of the SCW of this snail. Qualitative analysis of the SCW from *B. glabrata*, *H. trivolvis*, and *L. elodes* with mobile phase 1 verified that trehalose was not present. Five naphthol-positive zones below the maltose zone (R_f = 0.013, 0.060,

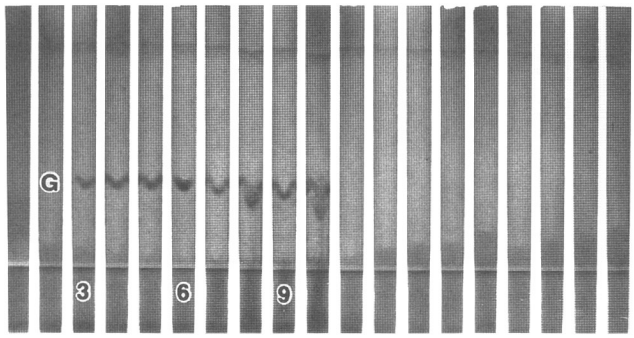

FIG. 1. Photograph of chromatograms used for quantification of glucose (G) in the mucus of *Lymnaea elodes*. Volumes of 3.00, 6.00, and 8.00 ml of the 100 ng/ml standard and 1.20 ml of the 1000 ng/ml standard were spotted in lanes 3–6. In lanes 7–10, 10.0-ml and 20.0-ml aliquots of two different mucus samples were spotted in adjacent lanes. Glucose zones of the 20.0-ml samples (lanes 8 and 10) were distorted due to overloading of sample in the preadsorbent area. Only the 10.0-ml glucose zones (lanes 7 and 9) were bracketed within the calibration curve generated by the CATS-3 software and used for quantitative analysis.

0.11, 0.15, and 0.22) were detected in all four strains of snails, but further analyses confirmed that these zones did not correspond to any of the sugar standards having R_f values lower than maltose, namely lactose, melezitose, raffinose, and trehalose [see Umesh et al. (1996) for a table of R_f values].

HPTLC analysis of sugars in two pooled mucus samples of *B. glabrata* and both strains of *H. trivolvis* displayed an amount of glucose below 0.0010%, the lowest quantifiable amount. Visual analysis of glucose zones showed darker bands in the *B. glabrata* samples than in *H. trivolvis*. Analysis of five *L. elodes* snails yielded quantifiable amounts of glucose, with a mean percentage from four trials of 0.0040% ± 0.00067% (mean ± SD). Standard and sample chromatograms are displayed in Figure 1 for the glucose analysis. Linear regression correlations (r values) for the calibration curves were 0.99–1.00.

DISCUSSION

Sugars were analyzed previously in the hemolymph and digestive gland–gonad complex (DGG) of *B. glabrata* (Anderton et al., 1993), Colorado and Pennsylvania strains of *H. trivolvis* (Conaway et al., 1995), and *L. elodes* (Cline et al., 1999a). Anderton et al. (1993) and Conaway et al. (1995) reported glucose and trehalose as the major sugars in the DGG and hemolymph of *B. glabrata* and both strains of *H. trivolvis*, while a similar study performed by

Cline et al. (1999a) on *L. elodes* showed glucose and maltose to be the major sugars present. Umesh et al. (1996) reported maltose instead of trehalose in the hemolymph and DGG of *B. glabrata* and both strains of *H. trivolvis*, contrary to the studies of Anderton et al. (1993) and Conaway et al. (1995). Cline et al. (1999b) have provided TLC and GC-MS identification of glucose and maltose in *B. glabrata*, and by using these techniques they were unable to confirm the presence of trehalose in this snail. Previous analyses showed that maltose is the major carbohydrate in *B. glabrata*, *H. trivolvis* (PA and CO strains), and *L. elodes*, and the present study revealed maltose to be the predominant sugar present in the SCW of these four types of snails. Sugar analyses in SCW have not been previously reported. Planorbid snails in the genera *Biomphalaria* and *Bulinus* are significantly attracted to chemical gradients of maltose as determined by means of diffusion olfactometers (Thomas, 1986; Kpikpi and Thomas, 1993). Thomas (1986) reported that the lower threshold of response by *B. glabrata* to maltose was between 5×10^{-6} and 5×10^{-7} M. It would not be surprising if maltose served as a chemical attractant for larval trematodes associated with planorbid and lymnaeid snails.

Mucus was analyzed by Wilson (1968) in the *L. truncatula* snail. Our study extends analyses to sugars in the mucus of *B. glabrata*, *H. trivolvis*, and *L. elodes*. It was more difficult to obtain adequate mucus samples from *B. glabrata* and the PA strain of *H. trivolvis* than from *L. elodes* because the planorbids did not secrete as much. Sufficient amounts for quantitative analysis could easily be obtained from the foot of *L. elodes* by stroking with a glass rod.

Wilson (1968) reported a concentration of 0.025% of glucose in fresh mucus of *L. truncatula* by using descending paper chromatography, compared to our value of 0.0040%. However, Wilson (1968), by using colorimetric means, reported that the glucose concentration was 2.26 mg/mg of fresh mucus in *L. truncatula*. Glucose has been implicated as a potential chemoattractant for *Schistosoma mansoni* to *B. glabrata* (Plorin and Gilbertson, 1985). Our study reaffirms Wilson's (1968) that glucose is a constituent of pulmonate mucus and as such is a potential chemoattractant for larval trematodes.

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UNPALATABLE COMPOUNDS IN THE MARINE GASTROPOD *Dolabella auricularia:* DISTRIBUTION AND EFFECT OF DIET

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Abstract—Sea hares are a rich source of novel secondary metabolites, most of which are derived from their algal diet, but the natural function(s) of these metabolites are largely unknown. We used field and laboratory assays to measure the palatability of extracts from the tissues, ink, and eggs of *Dolabella auricularia*. Digestive-gland extracts contained a wide variety of secondary metabolites, including the red algal compound prepacifinol epoxide and its derivative johnstonol, and they were unpalatable to reef fishes. Skin extracts were moderately unpalatable, but our bioassay-guided fractionation led us to (−)-7-dehydrocholesterol, rather than to an algal secondary metabolite. Ink extracts were consistently unpalatable to reef fishes only at high concentrations, suggesting either that ink must be concentrated to deter predators, that unpalatable components of ink rapidly decompose, or that ink has other functions. Unpalatability of ink was traced to a purple fraction, consistent with the hypothesis that the active compound is aplysioviolin, a known ink constituent modified from a red algal pigment. Egg extracts were moderately unpalatable; however, we could not trace this activity to any algal-derived secondary metabolite. Body-wall extract was highly palatable. Our results suggest that dietary-derived secondary metabolites play a role in chemical defense of *D.*

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auricularia via the ink, but are not responsible for unpalatability of skin or eggs. Accumulation of dietary-derived metabolites in the digestive gland may occur to detoxify a chemically rich diet, rather than or in addition to deterring predators.

Key Words—Anaspidea, chemical defense, dehydrocholesterol, *Dolabella*, johnstonol, prepacifinol epoxide, sea hare, secondary metabolites, sequester.

INTRODUCTION

Sea hares of the genera *Aplysia*, *Stylocheilus*, *Dolabella*, and *Bursatella* have provided natural-products chemists with a rich source of secondary metabolites, most of which are derived from the animals' algal diets (reviewed in Faulkner, 1992, 1997; Avila, 1995; Pettit, 1996; Yamada and Kigoshi, 1997). Despite interest in sea-hare chemistry dating back at least to Flury (1915), the function(s) of these secondary metabolites remains unclear (Pennings and Paul, 1993; Pennings, 1994; Nolen et al., 1995).

Since many algal metabolites deter feeding by herbivores, an obvious hypothesis is that sequestered compounds protect sea hares from potential predators (Carefoot, 1987; Faulkner, 1992). Although this hypothesis has some experimental support (Pennings, 1990; Paul and Pennings, 1991; de Nys et al., 1996), four lines of evidence indicate that the situation is more complex. First, sequestered secondary metabolites are located primarily in an internal organ, the digestive gland, rather than in the skin, where they could function more effectively in defense (Winkler, 1969; Faulkner, 1992; Pennings and Paul, 1993). Second, sea hare species that eat algae lacking in secondary metabolites appear to be just as unpalatable as species that eat chemically rich algae (Pennings, 1994). Third, individual sea hares raised on chemically rich and chemically poor algae do not differ greatly in palatability (Pennings, 1990; Nolen et al., 1995). Fourth, although sea-hare eggs appear to be unpalatable to predators (Faulkner, 1992; Pennings, 1994), attempts to isolate algal-derived compounds from them have failed (Faulkner, 1992; but see Carefoot et al., 1998), suggesting that eggs contain alternate lines of defense, perhaps compounds produced de novo by sea hares.

This evidence suggests that additional research is needed before an adaptationist explanation of sea-hare chemical defenses can be accepted. Here, we pursued three lines of inquiry. First, we compared the palatability of extracts from *Dolabella auricularia* tissues, ink, and eggs. Second, we compared the palatability of animals fed different algal diets. Third, we tried to identify unpalatable compounds to determine if they had an algal origin.

METHODS AND MATERIALS

Study Organism

Dolabella auricularia is a large (to ~1 kg wet mass), herbivorous, opisthobranch gastropod with a circumtropical distribution and a lifespan of ~16 months (Switzer-Dunlap and Hadfield, 1977, 1979). On the island of Guam (13°25′N, 144°55′E), *D. auricularia* occurs in shallow (<2 m) subtidal areas of mixed sand and boulders. Two morphotypes occur: a rare, relatively slender, agile morph and a common, relatively blocky morph, which we studied. *D. auricularia* is a generalized, nocturnal feeder (Pennings and Paul, 1992; Pennings et al., 1993) that sequesters secondary metabolites from algae (Faulkner, 1984, 1988; Pennings and Paul, 1993). *D. auricularia* and egg masses were collected by hand from Apra Harbor, Guam.

General Extraction and Feeding Assay Methods

Samples were extracted four times in 1 : 1 methanol-dichloromethane, and the organic layer was evaporated to dryness in a rotary evaporator. Residual solids were dried and weighed to calculate extract yields. Ink collected mixed with water was partitioned between methanol and dichloromethane to yield water-soluble and organic-soluble extracts.

Most feeding assays were conducted in the field on shallow (<5 m) reefs in Apra Harbor (Table 1). Fish that fed during our assays included: the damselfishes *Abudefduf sexfasciatus* and *Amblyglyphidon curacao;* the wrasses *Cheilinus fasciatus*, *Coris gaimard*, *Thalassoma hardwickii*, and *T. lutescens;* the triggerfish *Balistipus undulatus;* and the unicornfish *Naso vlamingii*. Extracts were incorporated into artificial diets consisting either of carageenan (4.0 g), paraffin wax (3.0 g), pureed frozen squid (2.0 g), and tap water (110 ml) (the squid diet) or of carrageenan (2.5 g), wax (2.0 g), Kruse Catfish Food (Kruse Grain and Milling, 5.0 g), and tap water (80 ml) (the pellet food diet). Ingredients were mixed, heated to boiling, and poured into a mold to form small (~1 cm^3) cubes. Experimental foods contained extracts or secondary metabolites dissolved in small amounts of solvent and added to the recipe after heating but before pouring; concentrations were based on the dry mass of the diet. Control foods contained identical amounts of organic solvent to control for any effect of solvent on feeding; however, the bulk of the solvent quickly evaporated from the food mixtures. Pieces of food were attached to 50-cm-long pieces of polypropylene rope (10 pieces/rope, spaced 3 cm apart for multiple comparisons, or 4 pieces/rope, spaced 5 cm apart for paired comparisons). Ropes were attached to the reef in groups of five to eight for multiple comparisons or in pairs (an extract versus a control). Ropes within a group or pair were spaced ~30 cm apart; different groups or pairs were spaced >2 m apart. All ropes in a group or pair were retrieved after fishes had removed

TABLE 1. FEEDING EXPERIMENT PROTOCOLS[a]

Assay	Design	Consumers	Diet	Treatments
November 1989 collection	MC	reef fish	squid	S, BW, DG, E, C, UC
October 1990 collection	MC	reef fish	squid	dorsal S, dorsal BW, foot S, foot BW, DG, E, I, C
Single diets	MC	reef fish	squid	S+BW, DG (*Dictyota* spp. diet), S+BW, DG (*Padina tenuis* diet), C, UC
Single diets DG	MC	reef fish	squid	DG (*Dictyota* spp. diet), DG (*Padina tenuis* diet), DG (field diet), C, UC
DG fractions	MC	reef fish	squid	C, 5 fractions
S fractions (June 1990)	MC	reef fish	squid	C, 5 fractions
S fractions (October 1990)	P	reef fish	pellet food	5 fractions versus paired C
S HPLC fractions	P	reef fish	pellet food	2 HPLC fractions versus paired C
Cholesterol	P	pufferfish	Prime Reef	Cholesterol versus paired C
(−)7-Dehydrocholesterol	P	pufferfish	Prime Reef	(−)-7-Dehydrocholesterol versus paired C
I	P	reef fish	squid	I versus paired C
I (two assays)	P	pufferfish	Prime Reef	I versus paired C
I MeOH-soluble fraction	P	pufferfish	Prime Reef	fraction versus paired C
I C18 fractions	P	pufferfish	Prime Reef	fraction versus paired C
E	P	reef fish	squid	E versus paired C
E fractions	MC	reef fish	squid	C, 5 fractions
E HPLC fractions	P	reef fish	pellet food	5 HPLC fractions versus paired C

[a]MC = multiple choice, P = paired choice, S = skin, BW = body wall, DG = digestive gland, E = eggs, I = ink, C = control, UC = unexposed control. All multiple choice assays had $N = 12$; sample sizes for paired assays differ for each comparison and are given in figures.

approximately half the total food pieces from that group or pair (1–10 min). For all multiple comparisons, cubes were weighed before and after the experiment to measure the proportion of food eaten. Unexposed controls included in several assays were handled identically to other ropes except that they were kept in mesh bags so that fish could not eat them. Results from unexposed controls indicated that changes in mass due to handling were minor relative to effects of feeding by fish. Proportional data were arcsine–square-root transformed before analysis. Results of multiple comparisons were analyzed by calculating all possible paired t tests and setting the critical P value equal to 0.05 divided by the number of tests. For paired comparisons, the number of food cubes completely eaten from each rope was scored, the data were analyzed with Wilcoxon's signed rank test, and the results reported as two-tailed P values.

Additional feeding assays (Table 1) were conducted in the laboratory by using the spotted sharpnose pufferfish *Canthigaster solandri* (Richardson 1844). *Canthigaster solandri* is an omnivore that feeds on a variety of filamentous algae and benthic invertebrates (Myers, 1989). We collected pufferfish from Pago Bay, Guam, by dipnet and held them individually in 4-liter flow-through containers submerged in water tables supplied with fresh running seawater. Laboratory experiments used a diet composed of carrageenan (2.5 g), frozen Prime Reef mix (35 g, Ocean Nutrition), and tap water (50 ml) (the Prime Reef diet). Individual pufferfish were offered one piece of food containing an extract or secondary metabolite and one piece containing only solvent. Paired trials without fish were conducted to estimate changes in the mass of food in the absence of a consumer. Individual replicates with fish and paired no-fish replicates were terminated after approximately half the total food was eaten (1–48 hr). Food pieces were weighed before the experiment, then blotted dry and weighed again after the experiment. Data were analyzed following Peterson and Renaud (1989): the variable "(change in mass of treated food) – (change in mass of control food)" was compared for replicates with and without fish by using a paired t test.

Distribution of Unpalatable Compounds

November 1989 Collection. Five *D. auricularia* and two egg masses were collected. The animals were dissected into three parts (skin, body wall, and digestive gland—other internal organs were discarded). Body parts and egg masses were extracted. Extract yields were typical of other collections except that the body wall had a relatively low yield (Table 2). Organic extracts were tested at natural concentrations (Table 1).

October 1990 Collection. Five *D. auricularia* and five egg masses were collected, and the *D. auricularia* were stimulated to release ink. The animals were dissected into five parts (dorsal skin, dorsal body wall, foot skin, foot body wall, and digestive gland—other internal organs were discarded). Body parts,

TABLE 2. EXTRACT YIELDS (% DRY MASS) FROM VARIOUS COLLECTIONS OF *Dolabella auricularia*[a]

	Field 11/89 (*na* = 5)	Field 6/90 (*na* = 12; *ne* = 19)	Field 10/90 (*na* = 5; *ne* = 5)	*Dictyota* diet (*na* = 4)	*Padina* diet (*na* = 3)
Digestive gland	24.4 ± 12.1	14.4 ± 1.6	31.7 ± 7.3	28.4 ± 12.1	29.0 ± 20.8
Skin	4.8 ± 1.2	6.4 ± 0.9			
Dorsal skin			9.9 ± 6.5		
Foot skin			6.1 ± 2.6		
Body wall	0.6 ± 0.1	3.5 ± 0.4			
Dorsal body wall			5.0 ± 2.0		
Foot body wall			2.3 ± 1.5		
Skin + body wall				1.7 ± 0.7	2.4 ± 0.3
Eggs		11.3 ± 1.0	28.4 ± 6.4		

[a]Data are means ± 1SD. *na* = number of animals extracted, *ne* = number of egg masses extracted.

eggs, and ink were extracted. Yields were typical of other collections (Table 2). Most organic extracts were tested at natural concentrations (Table 1). Because we had limited material, digestive gland and egg extracts were tested at concentrations of 25%, although their yields were slightly higher. Ink extract was tested at an arbitrary concentration of 25%.

Animals from Single Diets. Seven *D. auricularia* were collected and maintained individually in 40-liter aquaria with running seawater. Four individuals were fed a diet of *Dictyota* spp. (mostly *D. bartayresii*) and three a diet of *Padina tenuis* for 11 weeks, after which they were dissected into two parts (skin + body wall, and digestive gland—other internal organs were discarded) and extracted. Yields were within the range of other collections (Table 2). Extracts were tested at natural concentrations (Table 1).

To explore further if the diet that a *D. auricularia* had been eating affected the palatability of the digestive-gland extracts, we compared digestive-gland extracts from animals fed *Dictyota* spp. and *Padina tenuis* with extracts from animals eating a field diet (November 1989 collection), at a standard concentration of 10% (Table 1).

Sea Urchin Cleavage. We examined the toxic effect of different extracts from wild-caught animals and from animals fed single diets in a standard pharmacological assay. Extracts were incubated at 16 μg/ml with zygotes of the sea urchin, *Strongylocentrotus purpuratus*, in filtered seawater from the time of fertilization through the completion of the first cleavage in control zygotes (approx. 2.5 hr); controls were exposed to identical amounts of solvent. Results were recorded as percent change in division relative to controls.

Fractionation of Extracts

In order to attempt to isolate deterrent compounds from extracts of *D. auricularia*, a collection of 12 animals and ca. 40 egg masses was made in June 1990. *D. auricularia* were stimulated to release ink and dissected into three parts (skin, body wall, and digestive gland—other internal organs were discarded). Body parts, eggs, and ink were extracted. Extract yields (Table 2) were similar to other collections except that digestive glands and eggs had relatively low yields, perhaps because of seasonal variation or because extraction was not complete. Because the body-wall extract was highly palatable in previous assays, it was not tested further. Extracts of the digestive gland, skin, and eggs were separated into fractions of increasing polarity by using silica gel vacuum flash column chromatography [mobile phases: 100% hexanes, 25% ethyl acetate (EtOAc)–75% hexanes, 50% EtOAc–50% hexanes, 75% EtOAc–25% hexanes, 100% EtOAc).

Digestive Gland Fractions. Flash-column fractions from digestive glands were tested at a total concentration of 10% (Table 1). This concentration was lower than natural, but was used to encourage fish to feed because the crude extract of the digestive gland was extremely unpalatable at natural concentrations (see Results).

Skin Fractions. Flash-column fractions from skin were tested twice (Table 1). In the first assay, fractions from the June 1990 collection were tested at an overall concentration of 6% (slightly lower than the extract yield; Table 2). Based on the results of this assay, we fractionated the October 1990 dorsal-skin extract by using slightly different mobile phases (10% EtOAc–90% hexanes, 20% EtOAc–80% hexanes, 100% EtOAc, 50% EtOAc–50% methanol (MeOH), 100% MeOH). These five fractions were tested at an overall concentration of 9.9% (based on the extract yield; Table 2).

These two assays consistently identified the 20–25% EtOAc fraction as the only one deterring feeding (see Results). We isolated and tested (Table 1) two major HPLC fractions from this flash-column fraction by silica-gel HPLC (15% EtOAc–hexanes). Results indicated that a common sterol, (−)-7-dehydrocholesterol, was responsible for the unpalatability of the skin extracts. We purchased cholesterol (Sigma) and (−)-7-dehydrocholesterol (Aldrich) and tested these compounds at several concentrations (Table 1). Because (−)-7-dehydrocholesterol decomposes in the presence of oxygen and sunlight to give a variety of breakdown products, including vitamin Ds and sterol endoperoxides that are known to deter feeding (Bobzin and Faulkner, 1992), we cannot unambiguously assign the results of these assays to one particular compound. Based on TLC analysis, an unknown but large proportion of the (−)-7-dehydrocholesterol in our assays had decomposed by the time we returned uneaten food to the laboratory and reextracted it.

Ink Fractions. To determine if ink extract deterred feeding at lower con-

centrations than tested above (distribution of unpalatable compounds), we tested it in three assays at concentrations of 6.3, 5, and 5% (Table 1). Reasoning that ink might contain a water-soluble feeding deterrent, we partitioned ink between aqueous MeOH and dichloromethane, lypophilized the MeOH-soluble fraction, and tested it at a concentration of 32% (Table 1). Since ink organic extract deterred feeding in some assays, we obtained fractions using a reverse-phase C_{18} column eluted with MeOH, 50% H_2O–50% MeOH, 20% H_2O–80% MeOH, MeOH, 50% dichloromethane–50% MeOH, 100% dichloromethane, and 100% MeOH. The first two fractions were tested individually, while fractions 3–7 were pooled; all were tested at 4% (Table 1).

Egg Fractions. To confirm that extracts of eggs deterred feeding, we tested them at a concentration of 6% (Table 1). Flash-column fractions of eggs were tested at an overall concentration of 10% (lower than the extract yield, Table 2). Because only the 25% EtOAc–75% hexanes fraction deterred feeding, we further separated this fraction using silica-gel HPLC (5% EtOAc–95% hexanes mobile phase) into five HPLC fractions that we tested at an overall concentration of 6.3% (Table 1).

RESULTS

Distribution of Unpalatable Compounds

November 1989 Collection. Reef fish discriminated strongly among diets containing different extracts (Figure 1, top). Those with digestive glands and eggs were consumed significantly less than the control. Digestive-gland extract diet was so unpalatable that it did not lose any more mass than did the unexposed control. There was a nonsignificant trend towards the skin extract deterring feeding.

October 1990 Collection. Reef fish discriminated strongly among diets containing different extracts (Figure 1, bottom). Those containing digestive glands and ink were consumed significantly less than the control. There were nonsignificant trends towards the egg and skin extracts deterring feeding. Although body-wall extract was tested at a much higher concentration than in the previous assay, it still had no significant effect.

Animals from Single Diets. Extracts of digestive glands from animals fed either *Dictyota* spp. or *Padina tenuis* strongly deterred feeding by reef fishes (Figure 2, top). Body wall + skin extracts did not significantly deter feeding relative to controls. When extracts of digestive-glands from animals fed *Dictyota* spp., *Padina tenuis*, or a field diet were compared at a standardized concentration of 10%, all three extracts deterred feeding, but reef fish did not discriminate between extracts (Figure 2, bottom).

Sea Urchin Cleavage. Digestive gland extracts strongly inhibited cleavage

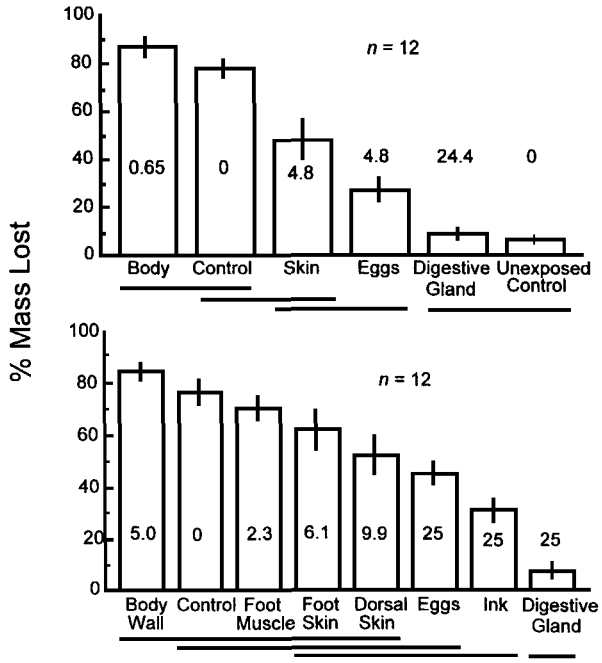


FIG. 1. Effect of extracts of *D. auricularia* on feeding by reef fishes. Data are means ± 1 SE; extract concentrations (% dry mass) are shown in or above bars. Horizontal bars connect means that are not significantly different. Top: November 1989 collection. Bottom: October 1990 collection.

of sea urchin zygotes, but extracts of skin, eggs, body wall, and ink had no effect (Table 3).

Digestive Gland Fractions

All digestive gland fractions deterred feeding, even at the relatively low total concentration of 10% (Figure 3). Fish did not significantly discriminate among digestive gland fractions. Examination by TLC indicated that all contained numerous compounds. Since all fractions deterred feeding and were chemically complex, we did not attempt a systematic study of which metabolites deterred feeding. However, we did isolate two secondary metabolites, prepacifenol epoxide and its derivative johnstonol, by HPLC. We targeted these because they were easy to purify from the complex extracts. They were also isolated from the digestive glands of animals that were fed *Dictyota* spp. in the laboratory (see Methods and Materials: Animals from Single Diets). Presumably,

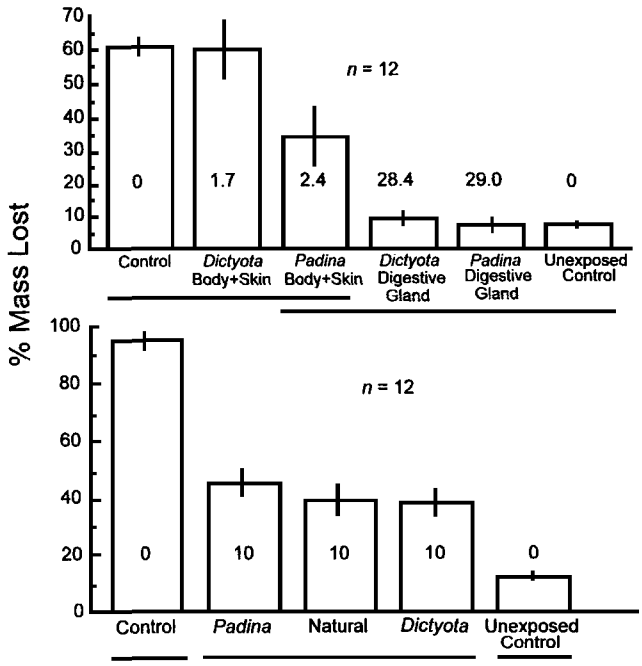


FIG. 2. Effect of extracts of *D. auricularia* on feeding by reef fishes. Data are means ± 1 SE; extract concentrations (% dry mass) are shown in or above bars. Horizontal bars connect means that are not significantly different. Top: animals fed single diets; natural concentrations. Bottom: digestive gland extracts from animals fed single diets and from November 1989 collection; identical concentrations.

TABLE 3. EFFECT OF EXTRACTS FROM *Dolabella auricularia* (16 μg/ml) ON ZYGOTE CLEAVAGE OF SEA URCHIN, *Strongylocentrotus purpuratus*

	Effect	Change (%)
Digestive gland (field diet)	inhibition	−67
Digestive gland (*Padina tenuis* diet)	inhibition	−73
Digestive gland (*Dictyota* spp. diet)	inhibition	−100
Skin	no effect	−7
Eggs	no effect	0
Body wall	no effect	0
Ink MeOH extract	no effect	0
Ink organic extract	no effect	0

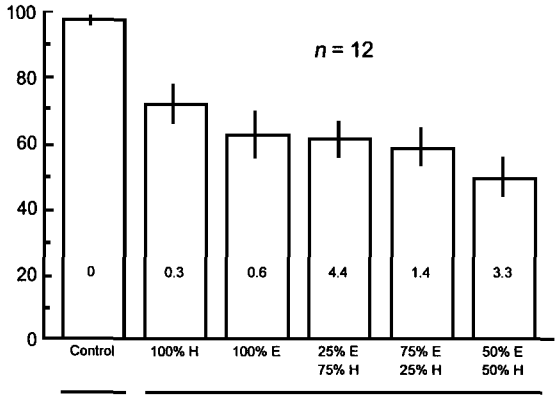


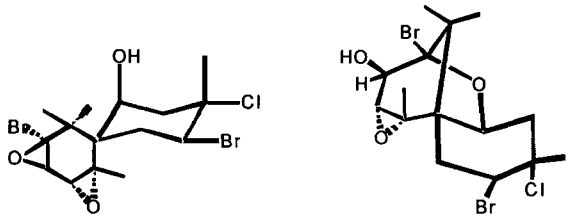
FIG. 3. Effect of digestive gland fractions (10% dry mass total concentration) of *D. auricularia* on feeding by reef fishes. Data are means ± 1 SE, extract concentrations (% dry mass) are shown in bars. Horizontal bars connect means that are not significantly different. H, hexanes; E, ethyl acetate.

they were eaten in the field before the experiment began and were slow to be excreted.

Final purification of prepacifenol expoxide and johnstonol was accomplished with HPLC by using a C_{18} column (5-μm Phenomenex Ultracarb 10 × 250 mm) and a gradient from H_2O to MeCN. Prepacifenol (**1**) rearranged to form johnstonol (**2**) over the course of time required to accumulate 2D NMR data (24 hr) in $CDCl_3$ (Scheme 1). The structure of johnstonol was supported by ^{13}C NMR and 2D NMR including COSY, HMQC, and HMBC experiments. Prepacifenol epoxide, physical data: ^1H NMR $\Delta \pm 0.05$ ppm from published values (300 MHz, $CDCl_3$) (Faulkner et al., 1974). Mass spectrum EI m/z (fragment, %): 442 (M^+, 2) (calcd. for $C_{15}H_{21}O_3ClBr_2$: 442); HREI m/z (fragment, %): 345.0242 (M^+-H_2OBr, 20) (calcd. for $C_{15}H_{19}O_2ClBr$: 345.0256, Δ 1.4 mmu). The physical data for johnstonol were comparable to published values. We also partially purified another digestive gland compound that appeared to be a dictyol diacetate.

Skin Fractions

The most deterrent fraction in each of the two sets of skin fractions was located in a region of similar polarity. In the first set, the 25% EtOAc–75% hexanes fraction was the least palatable (Figure 4, top). In the second, only the 20% EtOAc–80% hexanes fraction significantly deterred feeding (Figure 4, bottom). This fraction yielded two major mixtures after HPLC purification. Examination of these by NMR revealed that one was composed primarily of sterols (mostly



1
prepacifenol epoxide

2
johnstonol

SCHEME 1.

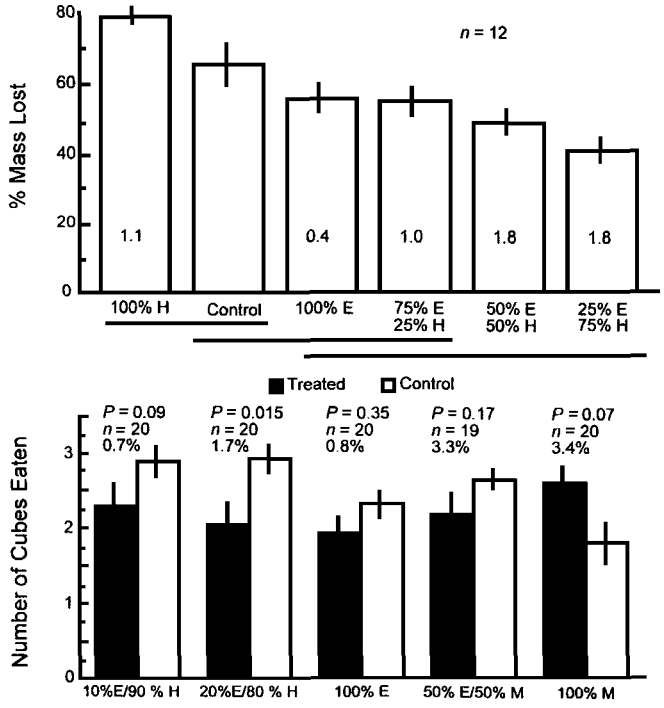
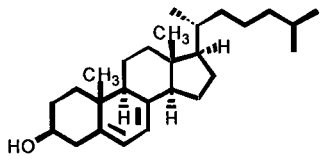


FIG. 4. Effect of two sets of fractions of extract of skin from *D. auricularia* on feeding by reef fishes. Data are means ± 1 SE. H, hexanes; E, ethyl acetate; M, methanol. Top: First set of extracts (6.1% dry mass total concentration); extract concentrations (% dry mass) are shown in bars. Horizontal bars connect means that are not significantly different. Bottom: Second set of extracts (9.9% dry mass total concentration); data are number of cubes out of four eaten. *P* values, sample sizes, and extract concentrations are given above bars.



3
7-dehydrocholesterol

SCHEME 2.

cholesterol) and the second of (−)-7-dehydrocholesterol. We compared the latter with an authentic sample of (−)-7-dehydrocholesterol (**3**) (Scheme 2) obtained from Aldrich (D300-1): $[\alpha]_D$ 100° (c = 0.0028 g/mL, MeOH), ^{1}H NMR $\Delta \pm 0.05$ ppm from standard material (300 MHz, $CDCl_3$); ^{13}C NMR $\Delta \pm 0.15$ ppm from standard material (75 MHz, $CDCl_3$). Trace amounts of the corresponding 5,8 endoperoxide were detected in the ^{1}H NMR spectrum, likely due to the oxidation of (−)-7-dehydrocholesterol.

The sterol fraction significantly stimulated and the (−)-7-dehydrocholesterol fraction significantly deterred feeding by reef fish (Figure 5). Cholesterol had no effect on feeding by pufferfish at a concentration of 1% (Figure 6). (−)-7-

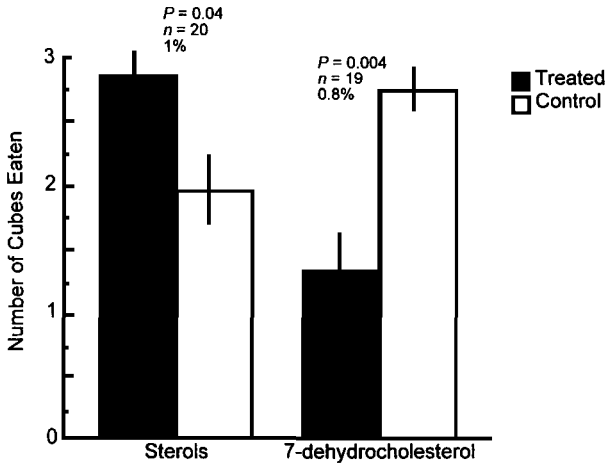


FIG. 5. Effect of two skin HPLC fractions [sterols and almost pure (−)-7-dehydrocholesterol] from *D. auricularia* on feeding by reef fishes. Data (means ± 1 SE) are number of cubes out of four eaten. *P* values, sample sizes, and extract concentrations are given above bars.

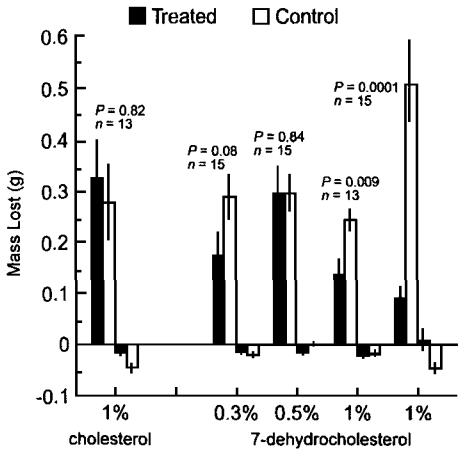


FIG. 6. Effect of pure metabolites cholesterol and (−)-7-dehydrocholesterol on feeding by pufferfish in the laboratory. Data (means ± 1 SE) are mass lost from diets exposed to (left two bars of each group) and not exposed to (right two bars of each group) pufferfish. *P* values and sample sizes are given above bars.

Dehydrocholesterol deterred feeding at a concentration of 1% in two different assays, but had no effect at 0.5 or 0.3% (Figure 6).

Ink Fractions

Although earlier tests with ink organic extract at a concentration of 25% indicated that it deterred feeding by reef fishes (Figure 1, bottom), effects at lower concentrations were inconsistent. Reef fish were not deterred by ink extract at 6.3% ($N = 17$, $P = 0.09$, trend towards stimulating feeding, data not shown). In one assay, ink extract at 5% had no effect on pufferfish feeding ($N = 8$, $P = 0.75$, data not shown); in a second assay it deterred feeding ($N = 7$, $P = 0.0016$, data not shown). The MeOH-soluble ink extract had no effect on feeding at 32% ($N = 11$, $P = 0.28$, data not shown). Of the three C_{18} fractions from the organic extract, only fraction 1 (MeOH) significantly deterred feeding by pufferfish (Figure 7). This fraction was purple; fractions 2 and 3–7 were off-white in color.

Egg Fractions

Egg extracts deterred feeding by reef fish when tested in a paired design at a concentration of 6% ($N = 18$, 0.67 ± 0.20 cubes with extract eaten versus 3.78 ± 0.13 control cubes eaten, $P < 0.001$). When the flash-column fractions were compared, only the 25% EtOAc–75% hexanes fraction, which comprised 64% of the total extract, deterred feeding (Figure 8, top). Of five HPLC fractions from

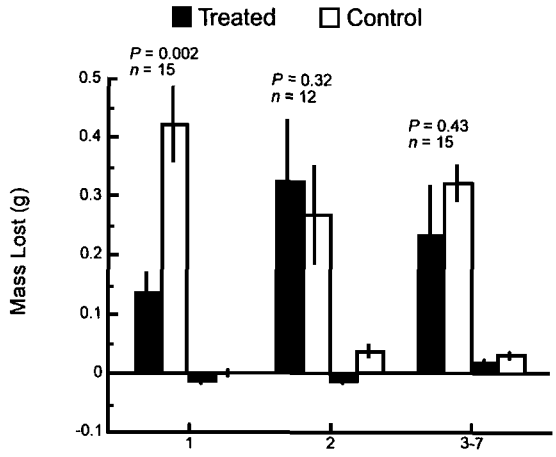


FIG. 7. Effect of fractions of extract of ink from *D. auricularia* on feeding by pufferfish in the laboratory. Data (means ± 1 SE) are mass lost from diets exposed to (left two bars of each group) and not exposed to (right two bars of each group) pufferfish. *P* values and sample sizes are given above bars.

this flash-column fraction, only one deterred feeding (Figure 8, bottom). This fraction had an orange–red color. A ^{1}H NMR spectrum of this active fraction suggested it was a triglyceride with unsaturated fatty acids.

DISCUSSION

Our results indicate striking differences in the secondary metabolite composition of various tissues, ink, and eggs from *D. auricularia*, and in their palatability. Extracts of digestive glands contained a variety of algal-derived secondary metabolites and were very unpalatable. Extracts of ink, eggs, and skin were unpalatable; however, the unpalatability did not appear to originate from algal compounds. Extracts of body-wall were palatable. Extracts of digestive glands inhibited cleavage of sea urchin zygotes, but extracts of ink, skin, body wall, and eggs did not. These results suggest that *D. auricularia* is chemically defended, but that these defenses involve more than just the utilization of dietary metabolites.

Digestive glands of *D. auricularia* contained numerous secondary metabolites, consistent with previous findings that *D. auricularia* is a generalized feeder (Pennings and Paul, 1992; Pennings et al., 1993). Although we did not identify all these compounds, we did isolate prepacifenol epoxide, known from the red algal genus *Laurencia* and the sea hare *Aplysia californica*, and its derivative

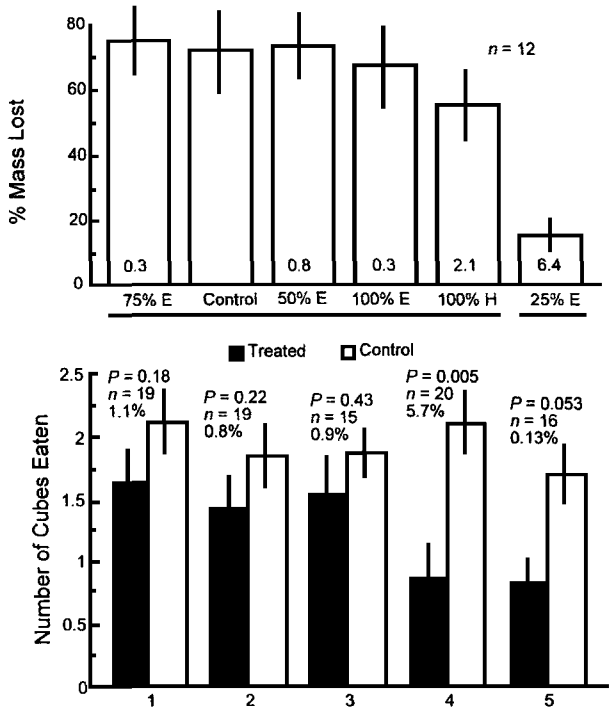


FIG. 8. Effect of fractions of extract of eggs from *D. auricularia* on feeding by reef fishes. Data are means ± 1 SE. H, hexanes; E, ethyl acetate. Top: Flash column fractions (10% total concentration); extract concentrations (% dry mass) are shown in bars. Horizontal bars connect means that are not significantly different. Bottom: HPLC fractions; data are number of cubes out of four eaten by reef fishes from paired treated and control ropes. *P* values, sample sizes, and extract concentrations are given above bars.

johnstonol (Sims et al., 1972; Faulkner et al., 1974; Ireland et al., 1976) and another compound that was probably a dictyol diacetate similar to a group of compounds known from the brown algal genus *Dictyota* (Faulkner, 1984). Other workers have isolated brown algal metabolites from *D. auricularia* (Faulkner, 1984, 1988). *D. auricularia* has also been a source of the promising anti-cancer compounds, dolastatins (Pettit, 1996), some of which probably originate from cyanophytes (Pettit et al., 1989).

Extracts of digestive glands were extremely unpalatable to reef fishes in our assays and strongly reduced cleavage of sea urchin zygotes. The digestive gland is the primary location of dietary-derived secondary metabolites in a variety of sea hares (Winkler, 1969; Faulkner, 1992; Pennings and Paul, 1993; de Nys et al., 1996). The simplest explanation for the accumulation of algal metabolites

in the digestive gland is that it is a way of detoxifying a chemically rich diet (much as the human liver removes a variety of toxic materials). An accumulation of unpalatable metabolites in an internal organ could not provide any selective advantage to *D. auricularia* through deterring predators since an individual *D. auricularia* would have to be disemboweled before the unpalatable metabolites could be detected. Consequently, even though an individual that sequestered secondary metabolites might be found distasteful by predators, it would not disproportionately survive to pass on its genes. However, if dietary-derived secondary metabolites were transferred to the skin, ink, or eggs, they might serve as a chemical defense. We evaluate this possibility below.

Although we found that extracts of skin were moderately unpalatable, our bioassay-guided fractionation did not lead us to an algal metabolite. Instead, we identified (−)-7-dehydrocholesterol as being responsible. This was surprising and may be an artifact since (−)-7-dehydrocholesterol decomposes to form vitamin Ds and sterol endoperoxides that could have been the source of the unpalatability. We do not know if this transformation would take place in vivo, nevertheless, no other skin fractions deterred feeding, suggesting either that the skin of *D. auricularia* is not unpalatable at all (i.e., all the skin results are artifacts), or that (−)-7-dehydrocholesterol is responsible for the unpalatability. (−)-7-Dehydrocholesterol is related to similar sterols isolated from sponges, tunicates, corals, and *Aplysia* spp. (Gunatilaka et al., 1981; Bobzin and Faulkner, 1992; Ortega et al., 1997; Slattery et al., 1997). Some of these have been shown to deter potential consumers (Slattery et al., 1997), indicating that similar compounds may be widespread in marine invertebrates, possibly playing an unappreciated role in defense against predators. Dietary-derived secondary metabolites are found in low concentrations in the skin of species of *Dolabella, Stylocheilus,* and *Aplysia* (Winkler, 1969; Faulkner, 1992; Pennings and Paul, 1993). Our results suggest that these compounds occur in *D. auricularia* at concentrations too low to deter feeding; however, they may be concentrated enough in some other sea hares to be a defense (Faulkner, 1992; de Nys et al., 1996).

The function of sea hare ink has been a long-standing mystery (Chapman and Fox, 1969; Nolen et al., 1995; Carefoot et al., 1999). The hypothesis that sea hare ink is unpalatable has been tested for *Stylocheilus longicauda* (Paul and Pennings, 1991), *D. auricularia* (Pennings, 1994), and a number of *Aplysia* spp. (Ambrose and Givens, 1979; DiMatteo, 1981, 1982; Pennings, 1994; Nolen et al., 1995) with ambiguous results. The results of our experiments are also ambiguous. Although organic extract of ink deterred feeding at a high concentration, it did so in only one of three assays when tested at lower concentrations. Since ink is rapidly diluted once released, our results suggest that ink could deter predators only at close range (e.g., if released directly into the mouth of a fish). Alternatively, it might be that ink contains unpalatable compounds that rapidly decompose and, hence, were absent in our experiments. Finally, ink might serve

a purpose other than antipredator defense or might deter predators by a mechanism other than bad taste (Carefoot et al., 1999).

The observation that the active ink fraction was purple and the two nonactive fractions were off-white is consistent with the hypothesis that the unpalatable material is the known ink constituent aplysioviolin, a monomethyl ester of the red algal pigment phycoerythrobilin (Faulkner, 1992). Thus, the possibility remains that sea hares incorporate dietary-derived compounds into their ink to deter predators. However, both the defensive nature of the ink and the identity of the unpalatable compound(s) remain to be established.

Our results indicate that eggs of *D. auricularia* contain an unpalatable, lipid-soluble compound or compounds. We did not completely purify this substance, but NMR analysis following preliminary purification suggested that it was more likely a triglyceride than a diet-derived algal secondary metabolite. Sea hare eggs are generally ignored by predators (Carefoot, 1987; Pennings, 1994), but efforts to isolate algal secondary metabolites from them have thus far failed (Faulkner, 1992). Work in our laboratory on eggs of *Aplysia juliana* also indicate that they are unpalatable and that the responsible compound is triglyceride-like in nature (Paul and Pennings, unpublished). Antimicrobial peptides and dietary-derived, mycosporine-like amino acids have been isolated from sea hare eggs (Kamiya et al., 1984; Carefoot et al., 1998), but it is unlikely that peptides or amino acids would have been extracted in the organic solvents we used.

If dietary-derived metabolites are important in defending sea hares from predators, we would expect that animals fed different diets would differ in palatability. Pennings (1990) raised small *Aplysia californica* on different diets and found that they differed in palatability to one species of predator, but not to others. By using a similar experimental design, Nolen et al. (1995) concluded that sea hare ink was an effective defense against predation by sea anemones but that secondary metabolites sequestered in the body were not. Moreover, there is little evidence that species of sea hares that eat chemically rich diets are less palatable than species that eat chemically poor diets (Pennings, 1994). In our experiments, the palatability of extracts from the digestive gland and skin of animals fed *Dictyota* spp. and *Padina tenuis* for 11 weeks did not differ from each other or from field animals. These results suggest that diet does not make a difference to the palatability of *D. auricularia*, at least on this time scale. Since diet-derived compounds may be retained in sea hare digestive glands for months (Stallard and Faulkner, 1974; Pennings and Paul, 1993), it is possible that maintaining animals on diets for a longer time period might alter their palatability.

Two important caveats apply to our results. First, it is possible that the process of dissecting animals and extracting the tissues might have introduced artifacts. Such artifacts could occur, for example, if bound chemicals were released, or if chemicals were not stable under the extraction conditions. If such artifacts occurred differentially in different extracts, our tests of relative palatability

could have been compromised. For example, ink might have contained a highly unpalatable but unstable compound that we could not detect with our methods. However, since our techniques were similar to those that have been used to isolate an endless array of algal metabolites from the digestive glands of various sea hares, it is unlikely that this potential artifact could cause typical algal secondary metabolites such as terpenes to be absent from the skin, eggs, or ink. Second, it is possible that our results might have differed had we used different species of consumers. This concern is difficult to address for organisms such as *D. auricularia* that have no known important predators since it is not obvious what the "correct" consumer to test would be. Most of our assays were conducted in the field with a diverse assemblage of fishes and therefore are unlikely to have been affected by the idiosyncratic behavior of any one consumer species. Nevertheless, it is possible that, for example, there could be compounds in the skin of *D. auricularia* that are highly unpalatable to crustaceans, but palatable to fishes.

Given these caveats, our results provide little support for the hypothesis that *D. auricularia* utilizes dietary-derived compounds to protect itself from predators. Dietary-derived compounds may make the ink of *D. auricularia* moderately unpalatable, but the role of ink in defense and the compounds involved have yet to be rigorously established. The skin and eggs of *D. auricularia* may be chemically defended, but the responsible compounds do not appear to be typical algal metabolites. *D. auricularia* does accumulate extremely high concentrations of algal secondary metabolites in the digestive gland, but this may serve to detoxify a chemically rich diet rather than to provide chemical defense.

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ATTRACTIVENESS OF OPPOSITE-SEX ODOR AND RESPONSES TO IT VARY WITH AGE AND SEX IN MEADOW VOLES (*Microtus pennsylvanicus*)

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Abstract—Current models suggest that among short-lived mammals, such as rodents, older adults are less attractive to the opposite sex and spend less time associated with the opposite sex than do younger adults. The objective of this study was to test two hypotheses in three different age groups of meadow voles. The first hypothesis is that 3- to 5 month old voles produce scents that are more attractive to opposite sex conspecifics than those of 7- to 12 month old and 14- to 18 month old voles. The second hypothesis is that 3- to 5 month old voles spend more time than either 7- to 12 month old or 14- to 18 month old voles investigating the scents of an opposite sex conspecific. The first experiment shows that when choosing between two conspecifics, females prefer the odor of the older male within each pair and that males prefer the odor of the 7- to 12 month old females to those of either 3- to 5 month old or 14- to 18 month old females. Thus, the data did not support the first hypothesis. The second experiment shows that the 14- to 18 month old males spent more time investigating female odors than did either the 3- to 5 month old or 7- to 12 month old males and that 7- to 12 month old females spent more time investigating male odors than did the 3- to 5 month old and 12- to 18 month old females. These data did not support the second hypothesis. Overall, older adult male meadow voles are more interested in and attractive to females than are younger adult males. The present data raise questions as to whether current models predict the age-related effects on the behavior of short-lived mammals.

Key Words—Meadow voles, scent, attractiveness.

INTRODUCTION

The literature is replete with theoretical and empirical evidence showing that age-related effects on reproduction in long-lived animals and short-lived animals dif-

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fer. Long-lived animals, such as large mammals, shore birds, and primates reach sexual maturity slowly, delay mating until well after reaching puberty, and then show a gradual, if any, decline in reproduction as they age; the postreproductive period or senescence occurs very late in the life-cycle (Clutton-Brock, 1988; Forslund and Part, 1995; Kokko and Lindstrom, 1996). In contrast, short-lived animals, such as rats, house mice, and voles reach sexual maturity rapidly, mate soon after reaching puberty, and show a dramatic decline in reproduction as they age; the postreproductive period occurs early in the life-cycle (Larsson and Essberg, 1962; Carr et al., 1970; Eliasson and Meyerson, 1981; Spruijt et al., 1989; Vega-Matusczyk et al., 1994).

The age of an individual will also affect the way conspecifics respond to it and how the individual responds to conspecifics. For long-lived birds and mammals, older adult males may be more attractive than younger adult males to females. Females may prefer to associate with and mate with older males because they are usually larger and more finely adorned than are younger adult males. Older adult males may be socially dominant, more experienced, or more interested in females than are younger adult males (Le Boeuf and Reiter, 1988; Gaillard et al., 1994; Nunn et al., 1994; Forslund and Part, 1995; Kokko and Lindstrom, 1996). Although less is known about how age affects these characteristics in short-lived animals such as song birds and small mammals, studies on laboratory strains of house mice and rats support the predictions of many models that these effects are opposite to those of long-lived animals. Senescent or near-senescent adult male rats and house mice are not as interested as recently postpubescent males in females or their odors (Spruijt et al., 1989; Hlinak and Krejci, 1990; Mencio-Wszalek et al., 1992). I am not aware of any studies that have directly examined whether these older adult males produce odors that are less attractive than those of younger adult males to females. However, based on the models of age effects on reproduction (Spruijt et al., 1989; Promislow, 1991; Rose, 1991; Boonstra, 1994), it can be inferred that older males likely produce odors that are less attractive to females than those produced by younger males.

For many rodents odor communication is an essential element of their social biology. Conspecific odors affect the reproductive physiology of individuals, and animals use odors to indicate their presence in an area and to signal their individual identity, willingness to mate, social status, and condition or quality to conspecifics (Johnston, 1983; Brown, 1985a,b; Halpin, 1986; Drickamer, 1992; Hurst, 1993). For example, in voles, the attractiveness of one's odors to conspecifics, its preferences for conspecific odors, and the way it responds to the scent marks of conspecifics are sexually selected traits that reflect some feature of the individual's quality and condition (Blaustein, 1981; Ferkin et al., 1995; Ferkin, 1999). Higher quality individuals, or those that are in breeding condition, are generally more attractive to, and attracted to, the opposite sex than are lower quality individuals or those that are not in breeding condition (Ferkin et

al., 1994, 1995; Solomon and Rumbaugh, 1997). Thus, the former individuals may be more likely than the latter individuals to announce their presence in an area and gain mating opportunities (Hurst, 1993; Ferkin, 1999).

In this study I test the generality of the prediction that among adult, short-lived, small mammals an individual's attractiveness to the opposite sex and its interest in the opposite sex diminishes with its age as it approaches reproductive senescence. The objective of this study was to determine whether the opposite sex attractiveness of the odor of meadow voles declines after reaching puberty and whether interest in the odor of an opposite sex animal declines in a similar manner. Specifically, I tested two hypotheses. The first hypotheses is that meadow voles in the oldest adult age group produce odors that are less attractive to opposite sex conspecifics than those produced by males in the younger two adult age groups. That is, males and females will spend more time investigating the odors of younger rather than older opposite sex conspecifics. The second hypothesis is that meadow voles in the oldest adult age group spend more time than voles in the two younger age groups investigating the odors of an opposite sex conspecific. That is, younger males and females will spend less time than older males and females investigating the odors of an opposite sex conspecific.

METHODS AND MATERIALS

Experiment 1

The purpose of this experiment was to determine whether the age of an adult meadow vole affects the attractiveness of its anogenital odor to opposite sex conspecifics housed under a photoperiod prevalent during the breeding season. During the breeding season adult meadow voles produce anogenital odor that is more attractive to opposite sex conspecifics than to same sex conspecifics (Ferkin and Seamon, 1987; Ferkin and Johnston, 1995; Ferkin et al., 1994, 1995).

Animals. Subjects and scent donors were male and female meadow voles (second- and third-generation laboratory-born voles) born and raised under long photoperiod (14L : 10D, lights on at 07 : 00 hr, CST and off at 21 : 00 hr, CST). Animals were weaned at 18 days of age, housed with littermates until 35 days of age, and housed singly thereafter in clear plastic cages (30.5 × 35.5 × 22.8 cm), containing cotton nesting material, water, and standard chow (Purina Mouse Chow #5015, PMI, St. Louis, Missouri).

Subjects were chosen from a pool of 63 adult females and 74 males. Under long-photoperiod conditions, meadow voles reach sexual maturity between 4 and 6 weeks of age (Dark et al., 1983). Subjects and scent donors were sexually experienced; however, females were not pregnant or lactating during the tests. All subjects were housed singly at least three weeks before the onset of the experiment.

Age Groups. Adult meadow voles were placed into one of three age groups: 3 to 5 months old (young), 7 to 12 months old (intermediate), and 14 to 18 months old (old). These age groups roughly correspond to the adult cohorts found in free-living vole populations (Brown, 1973; Christian, 1980; Negus and Berger, 1988; Sheridan and Tamarin, 1988; Negus et al., 1992; Slade, 1995). Voles 3 to 5 months old represent those animals born early in the breeding season. The 7- to 12 month old voles also represent those born during the breeding season, but overwintered as reproductively active adults (Gorman et al., 1993); together, these two age classes of voles make up the bulk of the population during the breeding season (Christian, 1980; Negus and Berger, 1988). Voles 14 to 18 months old represent those born the previous breeding season that have overwintered successfully, and continue on as reproductively active adults until the next winter (Negus et al., 1992). Voles in the latter age class comprise only a small part of the population (Brown, 1973; Negus and Berger, 1988; Negus et al., 1992). Voles in all three age classes are reproductively active and can successfully produce litters (Sheridan and Tamarin, 1988; M. Ferkin, S. Pugh, and R. Tamarin, unpublished data).

Experimental Procedure. Response to odor from the anogenital area of opposite sex conspecifics in each of the three age groups was evaluated. Subjects, 7- to 12 month old males and females, underwent a single odor preference test in which they chose between the scent of two donors. Scent donors were two conspecifics of the opposite sex. The subject was tested with either the anogenital area scent of a (1) 3- to 5 month old donor vs. that of a 7- to 12 month old donor, (2) 3- to 5 month old donor vs. that of a 14- to 18 month old donor, or (3) 7- to 12 month old donor vs. that of a 14- to 18 month old donor. Each subject was tested once and with a unique pair of scent donors; there were 15 different subjects used for each paired odor test. Scent donors and subjects were unfamiliar and unrelated to each other.

During the odor test, subjects were presented with anogenital area scent marks from two different donors on a clean glass microscope slide (2.5 × 7.6 cm). Fresh anogenital scent was collected for each trial by rubbing a sterile cotton swab against this area of a scent donor for 5–10 sec and then drawing the cotton swab across the slide. A different cotton swab was used for each scent donor. The first donor's mark was placed at one end of the slide in a streak (1 × 0.2 cm) in a horizontal direction. One minute later, the second donor's anogenital mark was placed at the other end of the slide in a streak (1 × 0.2 cm) in a horizontal direction. The placement and age of the first donor's scent mark and the second donor's scent mark on the right and left side of the slide were alternated, so that half the time the older donor's scent mark was placed first and the younger donor's scent mark was placed second and vice versa. The observer was blind to both the identity of the two scent donors and to the left- or right-side position of their scent marks on the slide. The middle section of the

slide contained no stimulus odors. Each slide was used for only one exposure and then discarded. The experimenter wore disposable latex gloves to minimize human scent transfer while handling slides.

Five minutes after the second donor's scent mark was deposited on the slide, the slide was suspended by a wire hook and clasp 1 cm above the substrate and against the wall opposite the nest in the subject's home cage; this caused minimal disturbance to the subject. The 3-min odor test began 10 sec after the slide was placed into the subject's cage. During the odor test, the subject was free to investigate the two scented portions and the clean, middle portion of the slide. Criteria for investigation of an odor were that a subject licked or sniffed a stimulus odor and/or its nose was directed toward and within approximately 1 cm of a scented portion. During the odor test, the amount of time spent investigating the two scented sections of the slide and the middle section of the slide was recorded continuously. However, the time investigating the middle section of the slide was not used in data analysis. Rather, it was used to compare the amount of time that the voles investigated the middle (clean) section of the slide with the time they spent investigating one of the two scented sections. Tests in which the subject spent more time investigating the middle portion of the slide than investigating the two scented sections were not included in the data analysis. Wilcoxon signed-ranks test were used to determine whether the age of the scent donor affected the amount of time subjects spent investigating the two stimulus odors; significant differences were accepted at $P < 0.05$ (two-tailed test).

Ten sexually experienced, 7- to 12 month old males, and 10 sexually experienced, 7- to 12 month old females were used as control subjects. In the controls, the amount of time that subjects spent investigating a clean glass slide was recorded.

Experiment 2

The goal of this experiment was to determine whether the age of meadow voles influences the amount of time they spend investigating the anogenital odors of older, opposite sex conspecifics housed under a breeding-season photoperiod. During the breeding season and under long photoperiod adult meadow voles prefer the anogenital odor of opposite sex conspecifics to those of the same sex (Ferkin and Seamon, 1987; Ferkin and Johnston, 1995).

Experimental Procedure. This experiment tested whether age affected the amount of time that 3- to 5 month old, 7- to 12 month old, and 14- to 18 month old male and female subjects spent investigating the anogenital area odor of a 7- to 12 month old opposite sex conspecific. Subjects were chosen from a pool of 73 females and 99 males. Several of the scent donors used in this experiment were subjects in experiment 1; however, no subjects used in this experiment were used as subjects in experiment 1.

In this experiment, we recorded the amount of time subjects investigated a slide containing the odor of a single, opposite sex donor. There were 15 different subjects in each treatment and each subject underwent only a single test. The odor testing procedure was identical to that detailed in experiment 1, except that the anogenital area scent of the single donor was placed on only one end of the slide in a streak (1×0.2 cm) in the horizontal direction; the middle section and other end of the slide contained no stimulus odors. The placement of the scent on the slide was alternated, so half the time it was on the left side of the slide and half on the right. During the 3-min odor test, the time that subjects spent investigating the scented section of the slide and the time spent investigating the remainder of the slide (unscented area) were recorded continuously. Only the time subjects spent investigating the scented portion of the slide is shown in the figures. Data were not used for analysis from any test in which the subject spent more time investigating the unscented portion of the slide than it did investigating the scented section. A one-way analysis of variance on ranks (Kruskal-Wallis H test) was used for each sex to assess significant differences in the time subjects spent investigating the odor of the opposite sex donors; significant differences were accepted at $P < 0.05$. When significant differences were found, I performed a posteriori Dunn's multiple paired comparisons to detect significant differences among age groups for males and for females ($P < 0.05$).

RESULTS

Experiment 1

All male and female subjects investigated the two sections of the slide containing the stimulus odors more than the middle (clean) section of the slide. In the control condition, voles spent little time investigating the clean glass slide (male subjects, $X \pm$ SE, 2.2 ± 1.2 sec and female subjects, $X \pm$ SE, 1.9 ± 1.0 sec); these times were roughly equivalent to those that male and female subjects spent investigating the middle section of the slide (male subjects, $X \pm$ SE, 1.4 ± 1.1 sec and female subjects, $X \pm$ SE, 2.1 ± 1.3 sec). Subjects did not display a left- or right-side preference in any test (χ^2 tests, $P > 0.10$; for all conditions).

Female subjects spent significantly more time investigating the anogenital area scent of the older male when choosing between the odors of two male donors. Specifically, females spent more time investigating the odor of 7- to 12 month old male donors than those of 3- to 5 month old male donors, the scents of 14- to 18 month old male donors than those of 3- to 5 month old male donors, and the scents of 14- to 18 month old male donors than those of 7- to 12 month old male donors (Figure 1A). These findings suggest that females prefer the odors of older males to those of younger males.

Male subjects spent significantly more time investigating the anogenital area

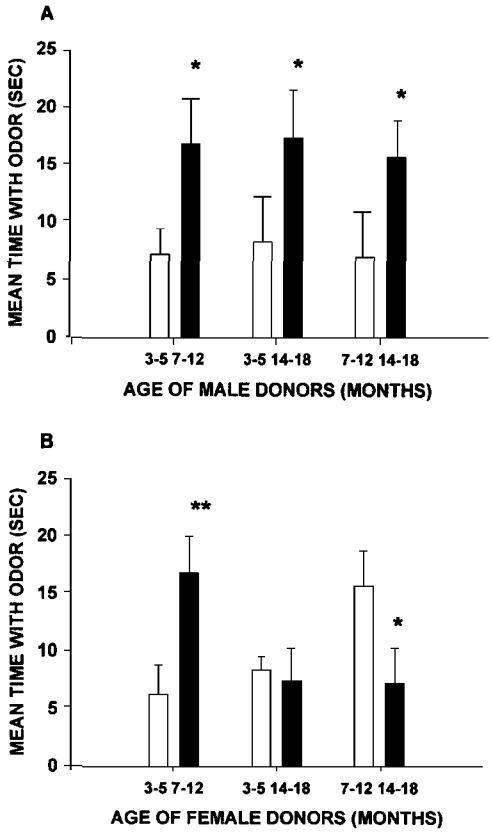

FIG. 1. The mean (± SEM) amount of time that female (A) and male (B) meadow voles, *Microtus pennsylvanicus*, investigated scent from the anogenital area of two scent donors of the opposite sex from the subject: 3- to 5 month old donor vs. 7- to 12 month old donor, 3- to 5 month old donor vs. 14- to 18 month old donor, and 7- to 12 month old donor vs. 14- to 18 month old donor. $N = 15$ subjects per paired comparison. Asterisks indicate statistical significance by Wilcoxon signed-ranks test ($^{*}P < 0.05$; $^{**}P < 0.01$).

scent of a 7- to 12 month old female when it was paired with the odor of either a 3- to 5 month old female or a 14- to 18 month old female (Figure 1B). These findings suggest that males prefer the odors of 7- to 12 month old females to those of younger or older females.

Experiment 2

All male and female subjects investigated the scented portion of the slide (the one containing the stimulus odor) more than the unscented portion of the

slide. Subjects did not display a left- or right-side preference in any test (χ^2 tests, $P > 0.10$; for all conditions).

The age of the male subjects affected the amount of time they spent investigating the odor of a 7- to 12 month old male [$H(2) = 19.175$, $P < 0.001$]. Males that were 14 to 18 months old spent significantly more time than either 3- to 5 month old males or 7- to 12 month old males investigating the anogenital area scent of a female scent donor (both comparisons, $P < 0.05$, Dunn's multiple pairwise comparisons, Figure 2A). The 7- to 12 month old and 3- to 5 month

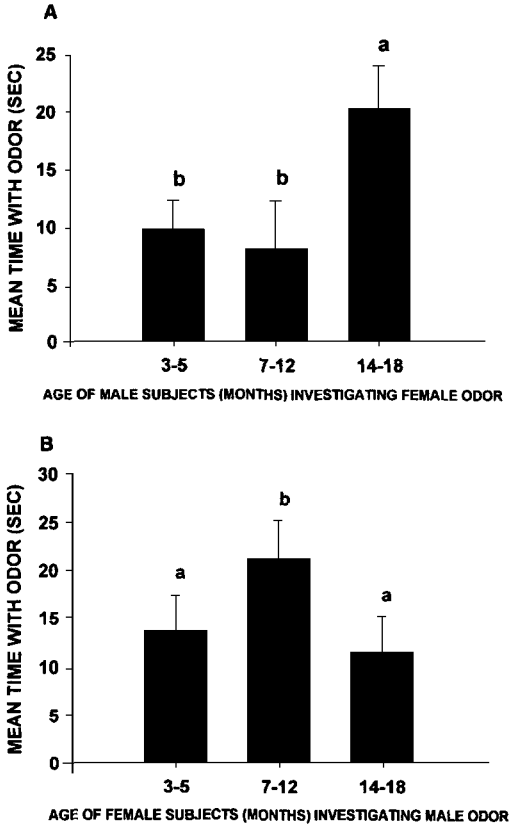

FIG. 2. The mean (± SEM) time that male (A) and female (B) meadow voles 3- to 5 months old, 7- to 12 months old, and 12- to 18 months old investigated anogenital area scent of a 7- to 12 month old opposite sex conspecific. $N = 15$ subjects per odor test. Statistical significance was determined by a one-way ANOVA on ranks followed by a posteriori Dunn's multiple pairwise comparisons. Histograms with different letters are statistically different from one another; significant differences accepted at $P < 0.05$.

old males spent similar amounts of time investigating the anogenital scent of a female donor ($P > 0.05$, ns, Figure 2A). The data suggest that the oldest males tested display the strongest preference for female scent.

The amount of time females spent investigating the odor of a 7- to 12 month old male was affected by her age [$H(2) = 28.973$, $P < 0.001$]. Female subjects to 7- to 12 months old spent significantly more time than either 14- to 18 month old females or 3- to 5 month old females investigating the anogenital area scent of a male scent donor ($P < 0.05$, Dunn's multiple pairwise comparisons, Figure 2B). The 3- to 5 month old female subjects and the 14- to 18 month old females spent similar amounts of time investigating the anogenital scent of a male donor ($P > 0.05$, NS, Figure 2B).

DISCUSSION

The results of experiment 1 clearly show that age affects the attractiveness of a male meadow vole's anogenital scent to females. Female meadow voles prefer the odor of an older adult male when that odor is paired with the odor of a younger adult male. This finding does not support the first hypothesis. Younger male voles did not produce odors that were more attractive than those of older male voles to females. The present data suggest that adult male meadow voles produce a scent that contains information about their age and that the scent of older males contains information about their longevity that is absent from the scent of younger males. Thus, females are selecting older adult males because these odors reflect a quality or specific feature of that male. Older males may have higher circulating testosterone or prolactin titers than do younger males; female voles find the odors of males with high testosterone and high prolactin titers more attractive than those of males with lower titers of either of these two hormones (Ferkin et al., 1994, 1997; Leonard and Ferkin, 1999). In this case, longevity may be an indicator of high quality males (Forslund and Part, 1995). Alternatively, females may prefer the odors of older adult males over those of younger adult males because they produce greater quantities of odiferous substances than do younger males. In male rabbits, house mice, and capybaras, older males have larger scent-producing glands and produce more odiferous substances than do younger males (Gonzalez-Mariscal et al., 1992; Herrera, 1992; Drickamer, 1995).

The data from experiment 2 show that older males spend more time than younger males investigating the anogenital odor of females. This suggests that odors of females may be more interesting to older males than to younger adult males. This finding does not support the second hypothesis. Young adult male meadow voles did not spend more time than old adult males investigating the odors of female conspecifics. The 14- to 18 month old males spent more time

than the 3- to 5 month old males and the 7- to 12 month old males investigating the odor of females. These findings suggest older adult male voles are more interested than younger adult male voles in the odors produced by female voles. However, it can be argued that because of captivity our voles do not represent aged individuals, and declines in the sexual attractiveness of their odors may not have occurred or may occur at a much later age. This is doubtful, in that age-related declines in male sexual behavior have not been reported in free living populations of voles (Sheridan and Tamarin, 1988; Slade, 1995). It is unlikely, therefore, that older adult male voles spend more time than younger males investigating females odors because the former males may have diminished sensitivity to female odors or lack the ability to readily distinguish among the odors of female conspecifics, as described for aged male rats and elderly humans (Doty et al., 1984; Spruijt et al., 1989; Corwin et al., 1995).

Interestingly, age had mixed effects on the odor attractiveness and odor preferences of female meadow voles. The results of the first experiment show that females in the 7- to 12 month old age group appear to be more attractive to males than females in both the younger and older age groups. This result provides only partial support for the first hypothesis in that 7- to 12 month old females produce scents that are more attractive than those of 14- to 18 month old females to males. Partial support was also gained for the second hypothesis in that 7- to 12 month old females spent more time than 14- to 18 month old females investigating the scents of males. Comparable age-related effects on behavior have been reported for 6- to 8 month old female gray-tailed voles (*M. canicaudus*). These female voles, which also reach sexual maturity at 4-6 weeks of age, displayed more proceptive and receptive behaviors and had a shorter latency to mate than did females that were either older and younger (Petersen, 1986). Similarly, studies on rats and ground squirrels indicate that females that are intermediate in age between puberty and senescence tend to be in better physical condition and produce more and heavier offspring than either younger or older female conspecifics (Dobson and Michener, 1995; Festa-Bianchet et al., 1995). Thus, like other female rodents in a comparable age group, 7- to 12 month old female meadow voles may be signaling their heightened willingness to mate and their fertility to male conspecifics.

Overall, the age-related effects on the attractiveness of male odors to females and the responses of males to female odors in meadow voles do not support the hypotheses that were tested and do not fit with those inferred from models of aging. Current models fail to predict the behavior of short-lived animals such as voles. The present data suggest that older male meadow voles may be trying to encounter females by producing odors that are attractive to females and by spending more time with females. It is not difficult to imagine that males that spend more time with females will have more mating opportunities compared to males that do not. This speculation is supported by field data on meadow

voles suggesting that older adult males tend to have greater reproductive success than younger adult males (R. H. Tamarin, S. R. Pugh, and M. H. Ferkin, unpublished data). An older adult male may provide females that choose him as a mate indirect and/or direct fitness benefits related to his longevity (Kokko and Lindstrom, 1996).

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EFFECT OF PREVIOUS EXPOSURE TO HYDROXAMIC ACIDS IN PROBING BEHAVIOR OF APHID *Sitobion fragariae* ON WHEAT SEEDLINGS

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Abstract—We hypothesized that aphids after previous exposure to hydroxamic acids (Hx), a family of secondary plant compounds deleterious to aphids, are able to reduce their subsequent exposure to them. This hypothesis was tested by evaluating the time to produce salivation into a sieve element (SSE) by the aphid *Sitobion fragariae* on seedlings of two wheat cultivars of *Triticum aestivum* differing in their concentration of Hx. The total time to produce a first SSE was significantly longer in the high-Hx cultivar; however, the subsequent, second SSE (first SSE after interruption of probing) in this cultivar was significantly reduced, reaching the level observed in the low-Hx plants. Therefore, a strategy to reduce the exposure to secondary compounds was observed only in the second SSE in high-Hx plants. When the experimental plant was replaced by a new unattacked plant after the first SSE, aphids did not change the behavior described, thus excluding an aphid-induced plant susceptibility. The number of cell punctures and accumulated duration was not affected by previous exposure to Hx, either in low or high Hx cultivars. Total time and pathway time but not cell punctures, seem to be the variables affected by previous exposure to Hx.

Key Words—Aphid, experience, probing behavior, sieve elements, salivation, cell punctures, hydroxamic acids, EPG, *Sitobion fragariae*, *Triticum aestivum*.

INTRODUCTION

Aphids are specialized feeders that probe through the plant epidermis and mesophyll in order to access their final feeding site, the sieve elements. Aphid

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probing behavior in both susceptible and resistant host plants has been reported frequently (Sharks and Chase, 1979; Montllor et al., 1983; Montllor and Tjallingii, 1989; Dorschner and Baird, 1989; Morgham et al., 1994, Webster et al., 1993; Caillaud et al., 1995). Probing behavior in resistant plants is characterized by shorter probes, longer nonprobing times, and shorter phloem ingestion times than in susceptible plants, suggesting that aphids are able to detect plant resistance factors and display aversive behavior.

In the cases of some cereals, the presence of hydroxamic acids (Hx) in the epidermis, mesophyll, and sieve elements (Argandoña et al., 1987; Niemeyer, 1991) has been suggested as the main resistance factor against aphids (Niemeyer and Pérez, 1995). Thus, aversive behaviors (Argandoña et al., 1993; Givovich and Niemeyer, 1995; Mayoral et al., 1996), as well as demographic parameters of aphid biology (Thackray et al., 1990; Niemeyer, 1991; Givovich and Niemeyer, 1994) have been correlated with Hx.

The mechanism by which Hx are recognized by aphids is rather speculative. In an intact plant, Hx are present as glucosides, which are enzymically hydrolyzed to aglucones when the tissue is injured and intracellular compartmentation is destroyed (Hofman and Hofmanova, 1971). Aglucones exert stronger effects on the behavior and performance of aphids than the respective glucosides (Corcuera et al., 1985). Damage to mesophyll cells by the stylets on their way to the sieve elements has been described based on light and electron microscopy studies (Pollard, 1973; Spiller et al., 1985; Tjallingii and Hogen Esch, 1993). All types of cells show punctures, and the highest numbers of damaged cells are found inside the vascular bundle, although the magnitude of cellular damage seems to be moderate (Brzezina et al., 1986; Tjallingii and Hogen Esch, 1993). There are no chemoreceptors present on the aphid labium or the stylets (Wensler, 1977; Tjallingii, 1978), and gustatory organs are located in the epipharyngeal wall and stimulated by ingesta (Wensler and Filshie, 1969). Hence, detection of secondary metabolites by aphids must occur through the sampling of plant contents.

The aim of this work was to study the effect of previous exposure to Hx on aphid probing behavior. Using the electrical penetration graph technique (EPG), we have chosen the time taken by an aphid to produce the first E1 waveform (first phloem phase) as our standard measurement. Time to E1 has been pointed out as an indicator of the location of non-sieve-element factors affecting plant acceptance by aphids (Tjallingii, 1995). E1 has been correlated with salivation into the sieve elements (SSE hereafter) (Prado and Tjallingii, 1994), which may be involved in suppressing the wound reaction of the sieve elements (Tjallingii and Hogen Esch, 1993). We hypothesize that after previous exposure of aphids to Hx during their path through the epidermis and mesophyll in their route to the sieve elements, they are able to reduce their subsequent exposure to them, particularly in high-Hx plants. We tested this hypothesis by evaluating the time

needed to produce a first SSE by the aphid *Sitobion fragariae* (Walker) on two wheat cultivars differing in their concentration of Hx and comparing it with the time needed to produce a subsequent SSE after interruption of probing. We also investigated whether differences in such behavioral responses are accounted for by the aphids' intrinsic probing ability or by changes in the plant's susceptibility induced by the aphid. With this aim, the experimental plant was substituted by a new unattacked plant after the first SSE by the aphid.

METHODS AND MATERIALS

Insects. S. fragariae were collected from wild annual Poaceae in central Chile. From a single collected individual, a colony of clones was created and kept on oats (*Avena sativa* L. cv. Nehuén) under laboratory conditions at 20 ± 2°C and 16L : 8D photoperiod.

Plants. Cultivars of the wheat *Triticum aestivum* L. differing in Hx concentration were used in order to expose aphids to plants with different levels of these plant secondary compounds. The first leaf of 7-day-old seedlings was used. Chemical analysis showed that Hx concentration in the first leaf was significantly higher in cv. Naofén than in cv. Millaleu [1.94 ± 0.31 mmol/kg fresh weight, $N = 8$, and 1.09 ± 0.30 mmol/kg fresh weight (mean ± SE), $N = 8$, respectively; F (1,14) = 29.21, $N = 16$, $P < 0.001$].

Experiments. In both wheat cultivars, probing behavior of the aphid was electronically monitored, and when a first SSE occurred (determined by the observation of an E1 waveform), the aphid was carefully separated from the plant and then immediately returned to it. Thus, the stylets were not allowed to stay in the lumen of the penetrated sieve element cell, and a new probing sequence was forced to start. The interruption lasted less than 5 sec. The experiment continued until a new SSE was observed (observation of a new E1 pattern) in the manipulated aphid.

A second set of experiments was performed, but in this case the experimental plant was substituted by another nonattacked plant once the aphid showed its first SSE. Such design allowed the control of a possible aphid-induced change in the plant due to the stylets' activities inside the plant tissues, a change that could be influencing the subsequent SSE. The plant used for replacement was of the same cultivar and developmental stage as the original plant. A total of 10 replicates in each experiment was performed.

Monitoring of Probing Behavior. The probing behavior of each experimental aphid was electronically monitored by electrical penetration graphs (EPG). A gold wire electrode (25 μm diameter) was fixed to the dorsum of an apterous adult aphid with conductive silver paint. Another electrode was inserted in the soil of the potted plant. Both electrodes were connected to a DC electric cir-

cuit designed to monitor aphid stylet incursions inside plant tissues (Tjallingii, 1978). When the aphid stylets penetrate into the plant tissues, they close the electrical circuit and the voltage changes are amplified and continuously monitored. All signals were directly displayed on screen and recorded on a PC hard disk for detailed analysis with the EPGview software with a sampling rate of 100 Hz (Flores et al., submitted). Different stylet activities and the location of the stylet tip produce specific patterns of voltage changes in the recorded signal (electrical penetration graph) so activity and tip position can be judged from the displayed signal (Tjallingii and Hogen Esch, 1993). With this technique, waveforms associated with nonprobing (NP), cell punctures (pds), pathway activities composed of intercellular–intramural advancement with salivary sheath formation (C), xylem ingestion (G), difficulties in the stylet during probing (F), and salivation (E1) in the sieve elements can be determined (Tjallingii and Hogen Esch, 1993).

Statistical Analysis. The times taken by aphids from their placement in the plant to the production of SSE (dependent variable) were compared with a three-factor ANOVA with repeated-measures analysis. Factors were labeled as Hx, SSEs, and Plant. Each factor had two levels. Factor Hx included cultivars with low and high Hx concentration; SSEs included the first and second SSEs; The Plant factor included conditions where the experimental plant was or was not substituted by another nonattacked plant once the aphid showed its first SSE. Non-probing time, pathway time, number of cell punctures, and their accumulated duration were similarly analyzed as dependent variables with ANOVA for repeated-measures. Tukey HSD test for multiple comparisons was also performed. Data were transformed by log $(x + 1)$ if ANOVA assumptions were violated.

RESULTS

Table 1 shows that the variation in the total time to produce an SSE was significantly affected only by the factor SSEs and by its interaction with the factor Hx (SSEs * Hx). Total time to produce an SSE significantly differed between first and second SSE and was also modulated by the Hx concentration. The Plant factor was not significant, indicating that plant substitution did not affect aphid behavior as measured by this variable. In the high-Hx cultivar, the total time needed to achieve a first SSE was significantly longer than the same period for the low-Hx cultivar (Figure 1A). Aphids showed a significantly shorter total time to achieve a second SSE with respect to the first SSE only in the high-Hx cultivar (Figure 1A). The time to achieve the second SSE was not significantly different in the two cultivars (Figure 1B).

Considering only the pathway time (C pattern) before an SSE as the depen-

TABLE 1. THREE-WAY ANOVA WITH REPEATED MEASURES OF TOTAL TIME TO PRODUCE SSE (DEPENDENT VARIABLE)

	Effect		Error			
Sources	*df*	MS	*df*	MS	*F*	*P*
Plant	1	0.046	36	0.099	0.472	0.496 NS
Hx	1	0.255	36	0.099	2.573	0.117 NS
SSEs	1	0.406	36	0.069	5.882	0.020
Plant * Hx	1	0.138	36	0.099	1.386	0.246 NS
Plant * SSEs	1	0.014	36	0.069	0.208	0.650 NS
Hx * SSEs	1	0.579	36	0.069	8.392	0.006
Plant * Hx * SSEs	1	0.104	36	0.069	1.513	0.226 NS

dent variable, the same trends were observed (Figure 1B). In this case, however, only the interaction SSEs * Hx was significant ($F_{1,36} = 4.62$, $P = 0.038$, three-factor ANOVA with repeated measures analysis). Similarly, for nonprobing time a significant source of variation was the interaction SSEs * Hx; however, this effect was accounted for by a significant difference in duration between nonprobing time before the first SSE in low- and high-Hx cultivars (14.3 ± 3.1 and 32.6 ± 6.6 min for low and high Hx, respectively, $P = 0.04$ Tukey HSD test). In contrast, patterns G and F did not show significant differences associated with plant substitution, Hx, or SSE (data non shown).

The number of cell punctures (pds) showed a significant effect only in the Hx factor ($F_{1,36} = 4.74$, $P = 0.036$, based on three-factor ANOVA with repeated measures analysis). Since the Hx * SSE interaction was not significant, this effect was due mostly to a larger difference between the number of cell punctures before the first SSE in low- and high-Hx cultivars [25.9 ± 4.17 and 53.8 ± 9.7 min for low and high Hx, respectively (mean $\pm$ standard error), $P = 0.04$, Tukey HSD test] than before the second SSE [31.8 ± 6.7 and 35.7 ± 5.7 low and high Hx, respectively (mean $\pm$ standard error), $P = 0.62$, Tukey HSD test]. The number of cell punctures was not different between the two periods before SSE within each cultivar (Figure 1C). The accumulated duration of cell punctures did not show significant differences associated with Hx or SSE (Figure 1D) or with plant substitution.

DISCUSSION

In the high-Hx plants, the first SSE was produced after a longer probing period than in the low-Hx plants. A similar significant effect of plant resistance on the times required to the first phloem access has recently been observed in a different aphid–plant combination (Chen et al., 1997). Interestingly, in the high-

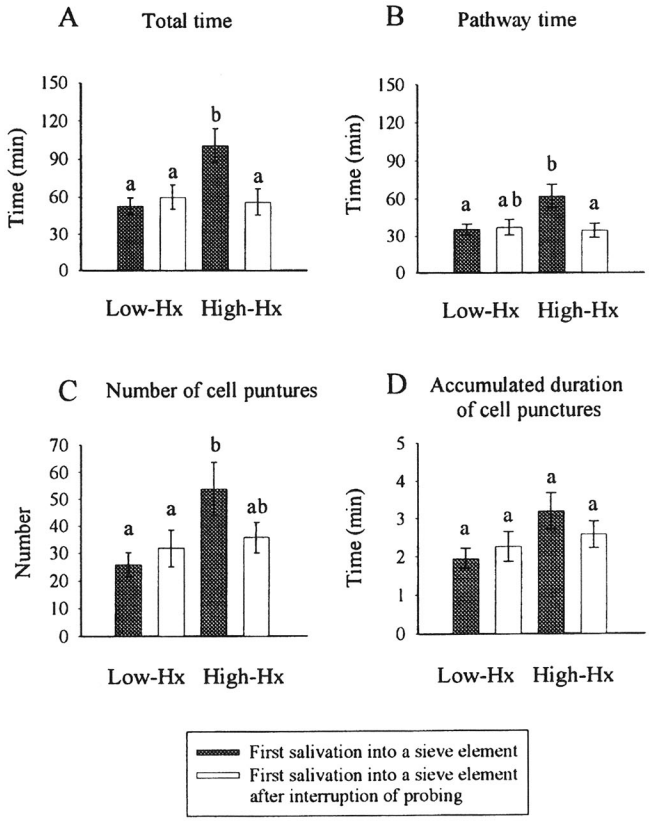

FIG. 1. Time to first SSE (dashed area) and to first SSE after interruption of probing (white area) by the aphid *S. fragariae* in wheat cultivars with low- and high-Hx concentrations. Total time before SSA (A), time in pathway activities (B), number of cell punctures (C), and duration of cell punctures (D) are shown. Bars represent standard errors for $N = 20$ in each case. Different letters among bars mean that the differences among the values are significant at $P < 0.05$ (Tukey HSD multiple-comparison test).

Hx plants, shorter times to the second SSE were recorded. Since time to achieve a second SSE in the high-Hx cultivar did not differ from the time devoted to the same activity in the low-Hx cultivar, it seems that aphids are able to overcome the high concentration of Hx during this period and consequently behave, at least in terms of their time assignation, as if they were on the low-Hx plants. Moreover, the significant decrease in the time to the second SSE in the high-Hx cultivar was attributable to a decrease in pathway activities but not to changes in other EPG patterns, suggesting that the main factor affecting plant acceptance is

encountered during passage through the intercellular and intramural plant compartments in the high-Hx cultivar. Furthermore, the fact that our experimental design did not allow phloem ingestion (E2 waveform) supports our proposal that prephloem ingestion factors are mainly involved in the behavioral changes described.

The fact that the decrease in the time to produce a second SSE was not affected by a substitution of the experimental plant for an unattacked one confirms that such time changes are accounted for by a change in the aphid's intrinsic ability to probe in the plant's tissues. It was not caused by an increase in plant susceptibility induced by the aphid during probing prior to its first SSE. This result is similar to the lack of aphid-induced plant effect on the behavior of the aphid *Rhopalosiphum padi* feeding on wheat (Prado and Tjallingii, 1997).

The number of cell punctures did not show significant differences between the first and second SSE in the two cultivars. Thus, it appears that interruption of feeding resets the pattern of cell punctures. This result is in agreement with those of Prado (1997), who found that the exploratory behavior of aphids seems to be reset by short interruption intervals (1 min). Total time and pathway time, but not cell punctures, seem to be the variables affected by previous exposure to Hx.

These facts support the hypothesis that after previous exposure to Hx during their route to the sieve elements, aphids employ a strategy of reducing their exposure to Hx, particularly when they face higher concentrations of them. This mechanism implies that during the first experience previous to salivation in sieve cells, aphids recognize the presence of Hx, particularly if present at a sufficiently high concentration. Such perception may initially elicit an avoidance behavior, mainly expressed as longer periods of pathway activities. This may be considered a training period within a simple learning process. Afterwards, during the path to a subsequent salivation into sieve cells, exposure to Hx is reduced through shorter duration of pathway activities as a result of the learning. An alternative explanation to the pattern observed is a mechanism of physiological adaptation to Hx by aphids, i.e., the deterrent effect of Hx decreases after exposure to them. However, because cell punctures may elicit hydrolysis of Hx from glucosides to the more toxic aglucones when the tissue is injured (Hofman and Hofmanova, 1971), the trend to show a lower number of cell punctures after the first SSE in the high-Hx cultivar seems to fit better a mechanism involving a reduction of the aphids' exposure to Hx.

Finally, the mechanisms proposed here should be understood as part of a variety of mechanisms that aphids may use in the field in order to overcome plant resistance factors such as secondary compounds.

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RECOGNITION OF DOMINANCE STATUS BY CHEMORECEPTION IN THE RED SWAMP CRAYFISH, *Procambarus clarkii*

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Abstract—We tested the hypothesis that chemical signals play a role in the recognition of dominance status in the red swamp crayfish, *Procambarus clarkii*. Dominance was judged on the outcome of dyadic interactions in all male or female groups of three individuals. This resulted in a dominant, intermediate, and subordinate individual within each population. A choice paradigm in a flow-through Y maze was used to judge whether crayfish were able to recognize dominance through chemical cues alone. Both individuals that interacted with the animal producing the odor and naive individuals were tested. Irrespective of sex and previous experience, individuals increased their rates of locomotion in the presence of conspecific odor. Naive males investigated the dominant arm first, spent more time at the dominant nozzle, and responded more aggressively (as measured by meral spread) to dominant male odor and subordinate female odor. Intermediate males spent more time at the dominant male nozzle and responded more aggressively to dominant male odor. Naive females spent more time at the dominant nozzle. These results show that males recognize dominant animals. Since both naive and experienced males respond to water from dominant animals, we concluded that this is recognition of dominance and not just individual recognition. This signal may be important for the formation or reinforcement of dominance relationships. Based on the change in behavior between odors, we suggest that crayfish can use chemical cues to recognize the dominance status of conspecifics.

Key Words—crayfish, *Procambarus clarkii*, dominance status recognition, chemoreception.

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INTRODUCTION

In some crayfish populations dominance status is important in shelter and food acquisition, as well as for reproductive success (Bovbjerg, 1953, 1970; Hazlett, 1979; Rutherford et al., 1995). An organism's status within a population is determined over time by agonistic interactions with population members (Huber et al., 1997). Physical characteristics such as body size and chelae size and number can affect the results of these interactions and the overall probability that an individual will have a dominant or subordinate status (Berrill and Arsenault, 1984; Rubenstein and Hazlett, 1973).

Several species of crayfish, including *Procambarus clarkii*, have been shown to establish dominance relationships (Bovbjerg, 1953; Copp, 1986). Crayfish behavior towards an individual is influenced by the outcome of past conflicts with that individual and possibly the other individual's status, which leads to shorter interactions between organisms (Rubenstein and Hazlett, 1973). It was originally thought that these subsequent behaviors depended upon learned individual recognition of dominant members by subordinate members (Bovbjerg, 1953; Hazlett, 1969). Copp (1986), using results from Thorpe and Ammerman (1978), suggests that it is not recognition of individuals that is occurring, but recognition of aggressive state. Since Thorpe and Ammerman (1978) did not quantify aggression or agonistic interactions, the only conclusion that can be drawn is that animals can detect two conspecifics in the same tank. So the debate as to whether it is individual recognition or recognition of an aggressive state occurring continues. Some authors suggest that recognition occurs through visual cues such as initiation or rate of escalation (Thorpe and Ammerman, 1978; Winston and Jacobson, 1978). If crayfish use an aggressive state as a recognition signal, it may be due to some aspect of the physiological state of a crayfish and not individual recognition (Copp, 1986).

Chemical signals are important for many crayfish behaviors such as foraging, predation, and mating. Crayfish use chemoreception to find food sources, regardless of the substrate type, through chemical cues by orientating to odor plumes emanating from food (Moore and Grills, 1998). Some chemical signals have also been shown to be alarm signals. Crayfish avoid areas where a conspecific has been injured or killed based on the chemicals released from an injured or dead conspecific (Hazlett, 1985a, 1990). In social situations, chemical signals allow crayfish to distinguish between male and female conspecifics (Ameyaw-Akumfi and Hazlett, 1975; Dunham and Oh, 1996) and to identify the correct species in mate choice (Tierney and Dunham, 1982). Ameya-Akumfi and Hazlett (1975) discovered that male *P. clarkii* can distinguish females from males through a putative sex pheromone. Dunham and Oh (1996) found the same behavior occurs in female *P. clarkii*.

Olfaction may be important for all these chemically mediated behaviors.

Although conclusive experiments have yet to be done, Ameya-Akumfi and Hazlett (1975) and later Dunham and Oh (1992) suggest that the sex pheromone is perceived primarily via chemoreceptors in the antennules of *P. clarkii*. The use of the antennules in agnostic interactions has been well documented (Smith and Dunham, 1996). In both light and dark environments, the rate of crayfish antennule flicking, an olfactory sampling behavior, during agnostic interactions is comparable (Bruski and Dunham, 1987). Dominant organisms exhibited an increased rate of antennule movement and duration of movement when compared to the rate and movements of a subordinate's antennule (Rutherford et al., 1996). The results of these studies suggest that crayfish are responding to chemical cues during conflicts and that these cues could play an important role in dominance status relationships.

Since both chemical communication and status relationships are integral parts of *P. clarkii*'s behavior, it is possible that there is a chemical signal associated with dominance status. We hypothesize that chemical signals play a role in dominance status recognition for the red swamp crayfish, *Procambarus clarkii*. This was tested by presenting both naive and "intermediate" crayfish with odors from dominant and subordinate individuals in a flow through Y maze that excluded all other sensory cues.

METHODS AND MATERIALS

Animals. Crayfish, *Procambarus clarkii*, were acquired from a commercial supplier (Carolina Biological Supply Company). They were separated by sex and housed in 10-gallon aquaria equipped with undergravel filters. Crayfish were maintained at a constant temperature of 23°C, a light–dark cycle of 14L : 10D, and fed 0.125-cm^3 pieces of frozen fish fillets (pollock, cod, herring, smelt) three times a week throughout the experimental period.

Population Structure. Populations of three same-sexed organisms were established. Dominance relationships among these organisms were examined using dyadic interactions that consisted of all possible pairwise combinations. Crayfish (carapace length ranging from 3.5 to 5.5 cm) were chosen randomly, marked with paint (Testors) on the carapace for individual identification, and introduced into a population tank at the same time. Male population tanks (78.7 × 78.7 × 31.8 cm) and female tanks (102.9 × 43.2 × 41.9 cm) contained five shelters (PVC pipe halves, 5 cm in diameter). Populations were allowed to interact for 72 hr, after which fight trials were conducted to determine dominance order. Tanks for males were larger than tanks for females because preliminary trials showed that aggression escalated to the point of death for males in smaller tanks.

Fight trials were performed in a glass test tank (50 × 25 × 30 cm) once a

day (09 : 00–12 : 00 hr) for each population. Animals participated in a single fight per day. Two crayfish were taken from the population tank and placed in separate opaque, plastic containers (28 × 18 × 13 cm). They were placed into the test tank at the same time and allowed to interact. The crayfish that tail flipped first was deemed the loser, and the other the winner. Previous work in our laboratory shows that tail flipping is the most obvious behavior exhibited by the loser at the end of the single encounters. More subtle behaviors, such as repeated retreats, are more reliable for repeated encounters between individuals. All fight trials resulted in a crayfish in each population with 2–0, 1–1, and 0–2 fight record. The crayfish in each population with a fight record of 2–0 was called dominant, 1–1 intermediate, and 0–2 subordinate. The tank, nets, and plastic holding bins were rinsed with distilled water for 2–3 min before each trial so that residual chemicals from the previous trial were eliminated. Naive males and females were isolated in separate opaque tanks (50 × 25 × 30 cm) for a week before being used in a trial. Populations were run through fight trials again after the Y maze study to ensure that the same hierarchy existed throughout the study period. Trials with population hierarchies that changed during the experiments or had crayfish molt during the experiments were removed from analysis (<4% of the populations).

Bioassay Arena. A flow-through Y maze (61 × 31 × 31 cm) was used for bioassays (Figure 1). This arena was large enough that animals could achieve full meral spread and turn around without striking the walls. A reservoir tank with two separate chambers (31 × 16 × 31 cm) supplied odor-laden water to the arms of the maze (Figure 1). All visual and mechanical stimuli between crayfish were eliminated using opaque paint on the outside of the tanks and opaque plastic wrap inside the tanks. Gravel was rinsed with distilled water and placed in each of the reservoir tanks and the Y maze before the start of a trial. Water flowed into the maze from the reservoir tanks through 1.0-cm-ID Nalgene tubing. The outflow pipes were 5 cm above the bottom of the maze. This height was chosen because it is the typical height of a crayfish nephropore, a potential source for chemical signals (Zulandt and Moore, 1998). Two in-line flowmeters (Manostat Riteflow #4) controlled flow. Dye trials, using commercial brand food coloring, were run at a rate of 168 ml/min to ensure that the flow from each holding tank was separate and equal when traveling through the arms of the Y maze.

Behavioral Trials. Dominant and subordinate crayfish from populations were randomly assigned to reservoir chambers A and B using a random number table (Zar, 1984). Crayfish were acclimated in the separate chambers for 3 hr to allow treatment crayfish odor to accumulate in the experimental water and to allow test crayfish to explore the Y maze without the influence of flow or odor. Treatments consisted of dominant and subordinate odors from a population presented together, dominant and no odor (blank), subordinate and no odor (blank), and no odor (blank) in either of the reservoirs (control). Experimental subjects were intermediate same sex, naive same sex, and naive opposite sex.

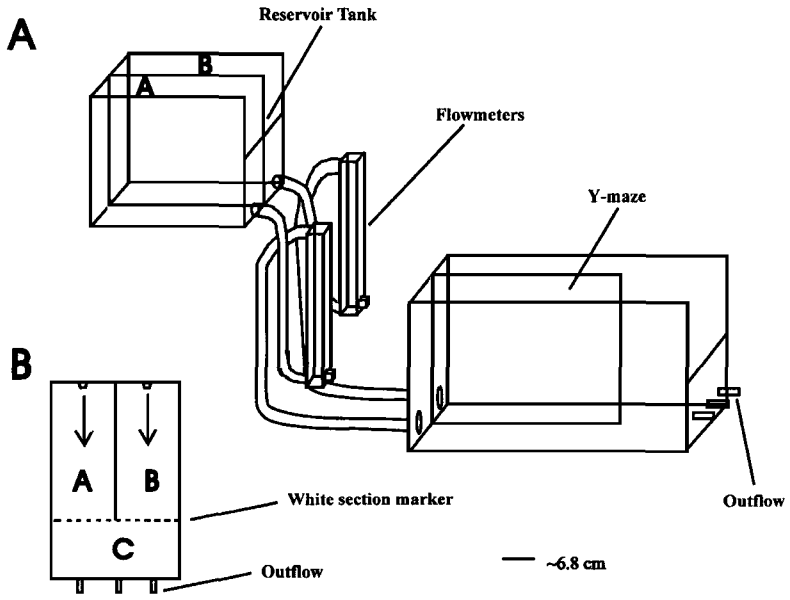

FIG. 1. Diagram of the flow-through Y maze showing the side view (A) and the overhead view (B). Water and odor in the reservoir tank flow to the same lettered sections in the Y maze.

At the start of each trial the flowmeters were set and maintained at a flow rate of 168 ml/min. A video camera (Panasonic wv-CL350) was set up 120 cm above the Y maze. The trials were recorded on a VCR (Panasonic AG-1980) and displayed on a monitor (Sony PVM-1351Q). All crayfish were returned to their respective population tanks after each trial. All experimental tanks and equipment were rinsed with distilled water before the start of each trial. Trials in which the test animal did not move more than one body length or in which the test animal was deemed visibly disturbed by the presence of the researchers were also removed from analysis (<12% of the total 88 trials were removed from analysis).

Data and Statistical Analysis. For each trial, initial arm choice, time spent in each arm, time spent at each nozzle, and meral spreads were measured by viewing videotapes. A crayfish was deemed in or out of an arm when its rostrum had crossed white lines painted on the top of the Y maze at the start of each arm (see Figure 1). The total amount of time spent in each arm was recorded, with time starting when the crayfish entered the arm and ending when it passed out of the arm. Time at the nozzle was defined as the total amount of time a crayfish spent directly in front of or touching the nozzle with at least one of its chelae.

Meral spread (distance between tips of chelae) has been used as a measure of aggression in many species by several investigators and was therefore used in this experiment (Thorpe and Ammerman, 1978; Bruski and Dunham, 1987). We measured meral spread at the beginning of the tank arm, 1/3 of the tank arm, 2/3 of the tank arm, and nozzle.

A binomial analysis was used to determine if there was an initial choice of Y maze arms different from random (Zar, 1984). A one-way ANOVA was used to determine if crayfish spent more time in any section of the Y maze in the absence of odor. These values were then used as expected values in a χ^2 analysis to determine if locomotory behavior changed with the addition of odor. Paired t tests were run to determine if one odor treatment was preferred over another in both total time in arm and time at nozzle comparisons (Zar, 1984). A repeated-measures nested ANOVA was used to analyze differences in meral spreads between odor treatments and arm choices. All statistics were run using a commercial statistics package (Statistica by StatSoft).

RESULTS

Effect of Odor on Time Spent in Y Maze Sections. In the absence of odor, both male and female crayfish spent significantly more time in section C of the Y maze (ANOVA: $F_{2,10} = 52.7$, $P < 0.001$; $N = 7$ for males and $N = 6$ females) (Figure 2). With the addition of odor, the preference of crayfish for a section changed, with a significant increase in time spent in the dominant and subordinate odor arms. Time spent in section C in the odor treatment significantly decreased. This was true for both male and female crayfish in all treatments.

Effect of Odor on Initial Arm Choice. Males show significant initial arm choice to only one of the conspecific odor treatments. Naive males chose dominant odor when paired with subordinate odor (binomial analysis, $P < 0.05$, $N = 9$) (Table 1). Females chose subordinate odor significantly more often when paired with no odor (binomial analysis, $P < 0.05$, $N = 6$) (Table 1). All other choices by females were not significantly different from random.

Effect of Odor on Time Spent at Nozzle. Naive males spent significantly more time at the nozzle of dominant animals of the same sex (Paired t test: $t_8 = 2.306$, $P < 0.05$, $N = 9$) (Table 2). Crayfish exhibited no differences in time spent at the nozzle in any other treatments.

Effect of Conspecific Odor on Meral Spread. Female crayfish did not exhibit a difference in meral spread size when exposed to any odor treatment (Figure 3B and C and Figure 4C). Naive male crayfish have larger meral spreads in the presence of subordinate female conspecific odor than dominant female odor (repeated-measures nested ANOVA: $F_{1,40} = 12.25$, $P < 0.01$, $N = 6$) (Figure 3A).

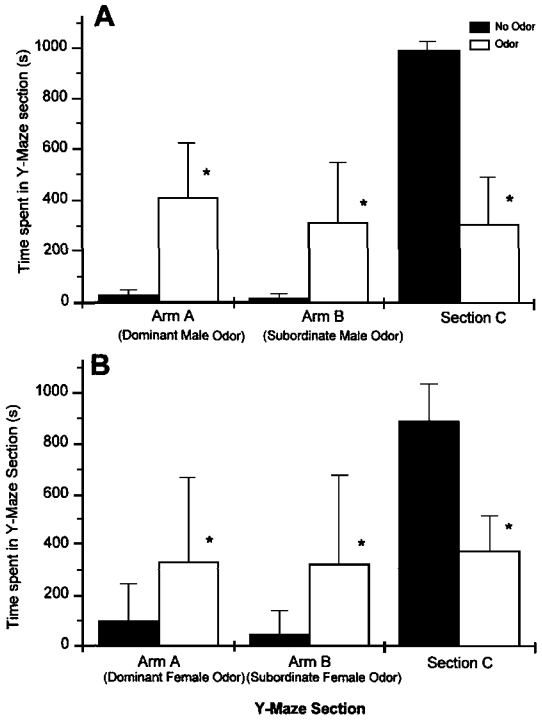

FIG. 2. The effect of male (A, $N = 7$) and female (B, $N = 6$) odor sources on crayfish time in Y maze sections. Black bars represent the no-odor treatment, and white bars represent the odor treatment. Bars are mean (+ SEM values) for the intermediate vs. dominant and subordinate odor trials. Asterisks mark significant differences between treatments at $P < 0.01$ using a χ^2 analysis. Both treatments were significantly different from the no odor treatment.

Intermediate males had a larger meral spread in the presence of dominant conspecific odor than subordinate conspecific odor (repeated-measures nested ANOVA: $F_{1,40} = 12.1$, $P < 0.001$, $N = 7$) (Figure 4B). Naive males had a larger meral spread in the presence of dominant conspecific odor than subordinate odor (repeated-measures nested ANOVA: $F_{1,52} = 24.31$, $P < 0.001$, $N = 9$) (Figure 4A). Intermediate males also exhibited a larger meral spread when presented with subordinate conspecific odor versus no odor (not shown) (repeated-measures nested ANOVA: $F_{1,32} = 5.547$, $P < 0.05$, $N = 7$). The subordinate odor induced meral spreads (mean ± SEM) of 3.71 ± 0.88 cm and 4.60 ± 1.12 cm at 2/3 the arm length and at the nozzle. The blank arm meral spreads were 2.31 ± 0.91 cm at 2/3 the arm length and 1.76 ± 1.00 cm at the nozzle.

TABLE 1. MALE AND FEMALE INITIAL ARM CHOICES IN RESPONSE TO CONSPECIFIC ODOR TREATMENTS

Sex and treatment	N	Percentage		
		Dominant arm	Subordinate arm	Blank arm
Male				
Intermediate male	7	33.3	66.7	
Intermediate male	7	50		30
Intermediate male	7		50	50
Naive female	7	50	50	
Naive male[a]	9	90	10	
Female				
Intermediate female	6	50	50	
Intermediate female	6	33.3		66.7
Intermediate female[a]	6		83.3	16.7
Naive female	6	50	50	
Naive male	6	66.7	33.3	

[a]Indicates a significant difference between arm choice in a treatment ($P < 0.05$, binomial analysis on raw data).

TABLE 2. AVERAGE TIME THAT MALE AND FEMALE CRAYFISH SPENT AT NOZZLE OF Y-MAZE ARM IN RESPONSE TO CONSPECIFIC ODOR TREATMENTS

Sex and treatment	N	Time (sec ± SEM)		
		Dominant nozzle	Subordinate nozzle	Blank nozzle
Male				
Intermediate male	7	15.83 ± 6.72	68.17 ± 59.07	
Intermediate male	7	7.83 ± 5.4		34.5 ± 32.54
Intermediate male	7		32.21 ± 15.9	3.2 ± 1.98
Naive female	7	33.33 ± 19.42	29.67 ± 13.61	
Naive male[a]	9	34.55 ± 16.3	1.11 ± 1.0	
Female				
Intermediate female	6	47.33 ± 26.44	54.5 ± 35.62	
Intermediate female	6	7.71 ± 3.86		6.83 ± 4.45
Intermediate female	6		8 ± 6.7	5 ± 3.64
Naive female	6	39.83 ± 21.2	11.5 ± 8.6	
Naive male	6	40.33 ± 22.6	36 ± 21.57	

[a]Indicates a significant difference between nozzle times within a treatment ($P < 0.05$, paired t test).

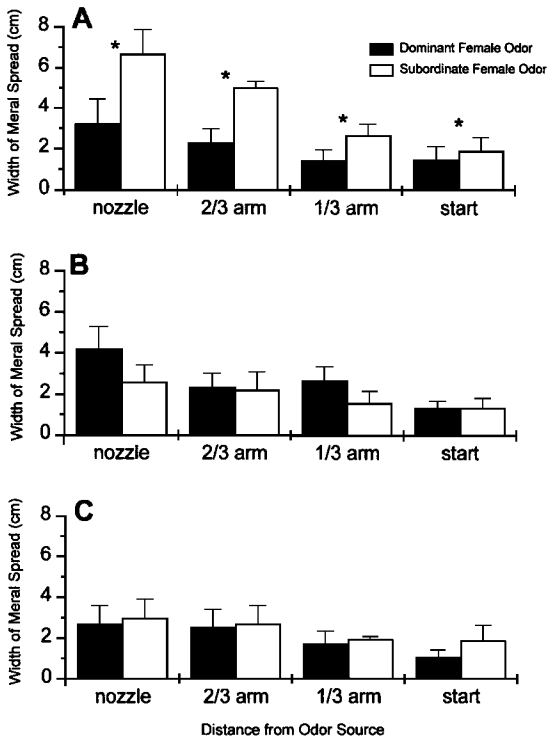

FIG. 3. The effect of female conspecific odor on naive male (A), intermediate female (B), and naive female (C) meral spreads to dominant (black) and subordinate (white) odors. Measurements were made at the nozzle, 2/3 the way up the arm, 1/3 the way up the arm, and at the start of each arm. Bars are mean meral spread widths (+ SEM); $N = 6$ for all trials. Asterisks mark significant differences between odor treatments at $P < 0.05$ using a nested ANOVA design.

DISCUSSION

Both male and female crayfish can detect conspecific odors. These odors cause a change in behavior that is reflected in the change in time spent in Y maze sections (Figure 2). The addition of odor caused the crayfish to become more active and to explore the arms of the Y maze.

Naive males recognized both male and female dominance status. This is evident in the naive male's change in behavior when conspecific dominant and subordinate odors were present. The initial choice of naive males was the dominant arm significantly more times than the subordinate arm (Table 1), and they spent significantly more time at the nozzle with same-sex dominant odor (Table

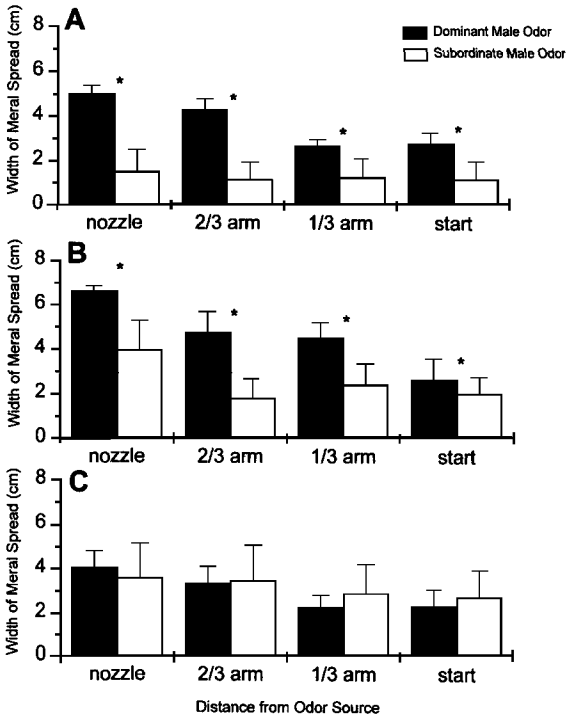

FIG. 4. The effect of male conspecific odor on naive male (A, $N = 9$), intermediate male (B, $N = 7$), and naive female (C, $N = 7$) meral spreads to dominant (black) and subordinate (white) odors. Measurements were made at the nozzle, 2/3 the way up the arm, 1/3 the way up the arm, and at the start of each arm. Bars are mean meral spread widths (+ SEM). Asterisks mark significant differences between odor treatments at $P < 0.05$ using a nested ANOVA design.

2). The naive males also had larger meral spreads in the presence of dominant male conspecific odor (Figure 4) and subordinate female odor (Figure 3).

Males that had previous exposure to test populations responded differently to water from a dominant male than from a subordinate male. Intermediate males had a larger meral spread in the presence of dominant male odor than in the presence of subordinate male odor (Figure 4B). Females that had previous exposure to test populations responded differently only to subordinate female odor (Table 1).

A possible explanation for differences in naive and intermediate results may be individual recognition. Previous exposure to a population results in different responses to odors from dominant and subordinate animals, but it is not clear whether this is due to individual recognition of the crayfish or recognition of a

status chemical by the experienced individual. The results from trials with naive animals support the idea that dominance status is recognized through chemical signals at least with naive individuals. Since all other cues have been eliminated, we conclude that naive females and males can recognize the hierarchical status of same-sex conspecifics through chemoreception (Tables 1 and 2, Figure 4). Naive males can also recognize the status of females (Figure 3). These signals could play an important role in the structure and maintenance of crayfish social communities.

There are several mechanisms by which dominance status could be recognized: a specific signal chemical, a unique mixture of chemicals, by volume of signal released, or the concentration of chemicals released. One mechanism could be through the detection of status-specific chemicals. Goldfish have a single chemical as an indicator of female sexual status (DeFraipont and Sorenson, 1993). The presence of this preovulatory pheromone causes males to increase sperm production in time for spawning (DeFraipont and Sorenson, 1993). Many organisms, however, use a blend of several chemicals for a single signal. Males of many moth species require a specific ratio of female mating pheromone chemicals to initiate mating behavior and to orient and find the female moth (Baker and Cardé, 1979; Akers and O'Connell, 1988; Liu and Haynes, 1993).

Another possible mechanism of recognition is through intensity differences between dominant and subordinate animals. A dominant crayfish could concentrate more chemicals or release more of the signal than a subordinate animal. If the signal is released in the urine, dominant animals may have the capacity to produce more urine, and therefore release a larger volume of urine than a subordinate animal would. This increased volume of urine produced by the dominant individual could play a role in the recognition of dominance status.

Status recognition through chemical cues has been documented in the cockroach. In these organisms, males produce a blend of three chemicals. The proportion of these chemicals emitted by a cockroach determines its hierarchical status to other cockroaches (Moore et al., 1997). Although hierarchical status recognition through chemical cues has not been studied in other systems, individual recognition has. Many rodents and insects have the ability to recognize individuals and conspecifics in their populations through a chemical cue (Johnston and Robinson, 1993; Hefetz, 1992).

Dominance status in the crayfish *P. clarkii* may also be under neural and hormonal modulation. Yeh et al. (1996, 1997) found hormone levels in crayfish changed depending upon the dominance status of the individual. Serotonin can modify the threshold of the tail flip (a sign of submission in agnostic interactions), suggesting the past fight history via changes in serotonin levels could play a role in dominance status recognition (Huber et al., 1997). This change in serotonin level may possibly be linked to the status pheromone in that it could be the entire chemical signal or one of its components.

Since recognition of dominance status does occur in our organism, the question arises as to whether the signal conveyed determines or reflects the status. In the cockroach, *Nauphoeta cinera*, the presence of the male sex pheromone determines rather than reflects the status (Moore, 1997). Thus, if the pheromone is produced, the organism is dominant; if not, it is subordinate. We would argue that in our system, the dominance signal most likely reflects rather than determines the status of the individual emitting it. It has been shown that the aggressive state of crayfish is closely linked to serotonin levels in the body and that these levels change depending upon the past fight history of an organism (Yeh et al., 1996, 1997; Huber et al., 1997). These levels can be reflected in released signals and therefore conceivably communicated to opponents. The signal therefore would not determine the status of the individual; rather it would reflect the success of encounters that dictates the dominance status.

Several authors have suggested that dominance recognition occurs through visual cues. Rubenstein and Hazlett (1973) showed that crayfish were able to recognize the aggressive state through visual displays of their chelae. They found a correlation between the area of the white meri displayed, aggression, and dominance. Copp (1986) also showed that visual cues were important in dominance status recognition. Both of these studies suggest a role for chemical signals, but none was able to determine conclusively if chemical recognition occurred.

In addition to visual cues, it seems plausible that benthic organisms such as *P. clarkii* would most likely have a status chemical signal as a consequence of the environment in which they live. Vision is not the only means of gathering information about the surrounding environment that is available to these animals. Crayfish use chemicals to recognize the sex of conspecifics and to avoid predation through the recognition of chemicals released from crushed conspecifics (Hazlett, 1985a,b). Because crayfish actively use chemical signals to acquire information about their environment, the use of chemicals in status recognition could be an invaluable form of communication for crayfish and play a large role in mate and resource acquisition.

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LEVELS OF ISOFLAVONOIDS AS INDICATORS OF RESISTANCE OF SUBTERRANEAN CLOVER TRIFOLIATES TO REDLEGGED EARTH MITE *Halotydeus destructor*

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Abstract—The extracts, obtained with methanol from crushed trifoliates and from the surface layer of the trifoliates, contained isoflavones in both the free and bound forms as the major components, compounds known to exhibit feeding deterrence towards the redlegged earth mite. The levels of these compounds were quantified and showed considerable variation in extracts of one susceptible variety and four varieties with differing levels of resistance. The relative resistance of the five varieties established from field observations was compared with the amounts of isoflavone derivatives obtained in the two extracts. A better ranking of resistance was found with levels of isoflavones on the leaf surface than with the total amount of isoflavones in the trifoliates. These results suggest a simple method for determining the relative resistance of subterranean clover to the mite.

Key Words—*Trifolium subterraneum*, trifoliate leaves, *Halotydeus destructor*, free isoflavones, bound isoflavones, plant resistance, deterrent activity.

INTRODUCTION

Redlegged earth mite, *Halotydeus destructor* Tucker (Acarina: Penthaleidae), is a destructive pest of pasture legumes, particularly of clover species (*Trifolium*

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spp), in southern Australia. The mites attack all stages of subterranean clover (subclover; *Trifolium subterraneum* L.), and the development of plant resistance is a priority in the management of this pest. In previous work, we have shown that resistance of subclover seedlings is positively correlated with the production of volatiles derived from lipoxygenation of fatty acids (Jiang et al., 1996a, 1997). Since the mites have access to the trifoliates for a much longer period of the season, we initiated a study of the resistance mechanism operating in trifoliates by using varieties identified as resistant to mite feeding (Gillespie, 1994; Ridsdill-Smith, unpublished data). Investigation of one resistant variety has shown that both the free and bound isoflavone derivatives present in the mature leaves exhibit deterrent activity to feeding by the mites, suggesting that the constituent isoflavones play a role in the resistance (Wang et al., 1998a). The free isoflavones, genistein, biochanin A, and formononetin, showed greater deterrent activity than the corresponding 7-*O*-glycosylated derivatives (Wang et al., 1998b). Consequently, it seemed important to determine the amounts and types of isoflavone derivatives in varieties of subclover that exhibit different resistance in the field. We report here on the levels of free isoflavones and their derivatives in the trifoliate leaves of five varieties with different levels of resistance. Since mites feed on the surface of the leaves (Ridsdill-Smith, 1997), we also determined the distribution of these compounds on the leaf surface of these varieties. The ranking between amounts and types of isoflavones with resistance is discussed.

METHODS AND MATERIALS

Plant Material. Seeds of subclover varieties (SE011, SE014, SE016, SE020, Dalkeith) sieved to constant weight were sown in pots with standard soil mix (Ridsdill-Smith and Gillespie, 1993). The pots were moved to a glasshouse after seed germination. The mature leaves (four to five weeks after seeding) were used for the experiments and analyses.

Redlegged Earth Mites. Mites were cultured in the laboratory on vetch (*Vicia sativa* cv. Blanchefleur) (Leguminosae) in summer and collected from pasture near Perth in winter. Mites were staged, and young adults, starved for 2 hr in a humid vial (15°C), were used for experiments.

Extract Preparation. Whole leaves were extracted for bioassay. For the preliminary choice tests, fresh leaves of different varieties were treated with liquid nitrogen, powdered, and steeped in MeOH at room temperature overnight. The residue obtained after removal of the solvent from the MeOH solution was used directly in the bioassays. Surface extracts were prepared for bioassay and HPLC. Fresh trifoliate leaves of the different varieties were dipped in MeOH for 10

sec, conditions that do not remove chlorophyll or carotenes (Hashidoko et al., 1992), and the solution was concentrated in vacuo to give a residue that was used for the bioassay and HPLC analysis. Typically, fresh leaf material (27 g) after dipping into MeOH yielded a pale yellow extract (14 mg). Whole leaves were also extracted for HPLC. The treated leaves were frozen with liquid nitrogen, powdered, and extracted with MeOH (×4) at room temperature. The combined methanol solutions were concentrated in vacuo to yield a residue that was analyzed by HPLC. Typically, a dark green residue (0.9 g) was obtained from the leaves (27 g) on total extraction with MeOH.

General Analytical Methods. For HPLC, an HP 1050 instrument equipped with an HP ODS hypersil column (5 mm, 250 × 4 mm) was used with the detector set at 260, 270, and 280 nm. The eluting solvents used (1.0 ml/min) were water (2% AcOH) (A) and MeOH (B) in the following gradient: 20% B in A increasing linearly to 70% B over 15 min. This was maintained for 5 min, and then the concentration was linearly increased to 100% B over 5 min and held at this concentration for 5 min. The amounts of analytes in the extracts were determined by using internal standard procedures based on a calibration curve obtained from duplicate injections of genistein at five concentrations. The values given are the mean of three replicates.

Assay for Feeding Deterrence. Extracts from trifoliate leaves were bioassayed for feeding deterrence towards redlegged earth mites. The membrane sachet technique previously described by Jiang et al. (1996b) was used. Mites were given a choice of a sachet containing a feeding stimulant (1% aqueous glucose) with or without a treatment sample from Dalkeith, SE020, SE016, and SE014. The fifth variety, SE011, was not tested in the bioassay. Membrane sachets were made with stretched Parafilm. For each sachet, a membrane was stretched over a 2-cm-diameter plasic ring, 35 ml of test solution was added, and a second membrane was stretched over the top. In choice experiments, two membrane sachets were buried, nearly touching, so as to be just above the surface of the soil (4 : 1 sand to loam), which was moistened close to field capacity, in a three-quarters filled plastic jar. Twenty mites were released by gently tapping them from a vial onto the soil beside the membrane sachets in the plastic jar, which was then sealed with stretched parafilm to contain the mites. There were 20 replicates for each experiment. Experiments were carried out at 15°C under fluorescent light in a room with no natural light. Mites were observed through the parafilm membrane and the number of mites on each membrane sachet was counted at 20- or 30-min intervals for 3 hr. Deterrence in the choice test for each experiment was calculated from the average of seven observations as follows: [(mite number on control − mite number on treatment)/total number of mites] × 100. A positive value is taken to refer to deterrence. The mean of seven observations during 3 hr is presented. Differences between means were examined with paired t tests.

RESULTS

Bioassays of Total Extract from Leaves of Subclover Varieties. Bioassays of the methanol extract obtained from three of the resistant subclover varieties showed that the extracts had significant and comparable feeding deterrence to the redlegged earth mites; SE020 (69%), SE014 (56%), and SE016 (61%) compared to a sugar control (Table 1). The extract from the susceptible variety Dalkeith was not significantly deterrent. A pairwise choice test of the activity of the extracts compared to that from Dalkeith showed that those from the resistant varieties were all significantly deterrent (Table 2). The extract from SE014 was most deterrent and that from SE016 the least.

Bioassays of Leaf Surface Extracts from Subclover Varieties. Extracts prepared by simple washing of the leaves with methanol were also tested for feeding deterrence. The methanol extracts from the varieties SE014, SE016, and SE020 and Dalkeith showed deterrence at 1% concentration compared to a sugar control, although the deterrence of the extract from the variety Dalkeith was half that of the resistant varieties (Table 3). At 0.5% concentration, deterrence was lower for all treatments and was significant for SE020 only. Significantly more mites were found on the Dalkeith extract than the control.

Quantitation of Levels of Isoflavone Derivatives in Total Methanol Extract and on Leaf Surface. The extracts obtained by methanol extraction of the leaves of the susceptible (Dalkeith) and four resistant varieties (SE011, SE014, SE016, and SE020) were analyzed by HPLC. The compounds monitored were biochanin A, genistein, formononetin, their corresponding 7-*O*-glucosides, and the 6″-malonate-7-*O*-glycosides of biochanin A (BGM) and genistein (GGM), which have been shown previously to be the major extractable constituents of the leaf (Wang et al., 1998a,b). Table 4 lists the levels found for these compounds. The resistant varieties contained 1.5–6 times more free isoflavones and 6–150 times more glucosylated isoflavones.

TABLE 1. MEAN NUMBER OF MITES IN PAIRWISE CHOICE TEST ON CONTROL AND TREATMENT MEMBRANE SACHETS CONTAINING METHANOL EXTRACTS (3%) FROM VARIETIES OF SUBCLOVER AND CONTROL WITH NO EXTRACTS OVER 3 HOURS[a]

Variety	Control (mean ± SE)	Treatment (mean ± SE)	Deterrence (%)	*P*
Dalkeith	4.66 ± 0.645	2.45 ± 0.462	31.1	0.060
SE016	7.81 ± 0.884	1.87 ± 0.283	61.4	<0.001
SE020	7.94 ± 0.722	1.47 ± 0.234	68.7	<0.001
SE014	9.78 ± 0.665	2.77 ± 0.340	55.8	<0.001

[a]Both control and test membrane sachets contained 1% glucose and 5% Tween 80.

TABLE 2. MEAN NUMBER OF MITES IN PAIRWISE CHOICE TEST WITH METHANOL EXTRACTS (3%) FROM VARIETIES OF SUBCLOVER AND DALKEITH OVER 3 HOURS[a]

Choice	Character to mite[b]	Control (mean ± SE)	Treatment (mean ± SE)	Deterrence (%)	*P*
Dalkeith–SE016	S-R	4.93 ± 0.938	2.66 ± 0.364	29.9	0.039
Dalkeith–SE020	S-R	4.88 ± 1.006	2.37 ± 0.451	34.6	0.015
Dalkeith–SE014	S-R	4.06 ± 0.641	1.55 ± 0.288	44.7	0.014

[a]Both control and test membrane sachets contained 1% glucose and 5% Tween 80.
[b]S = susceptible; R = resistant.

The levels of the same compounds in the extracts obtained from the surface of the leaves were determined in a similar fashion and are given in Table 5. Levels are lower than for the extract from whole leaves.

DISCUSSION

The bioassays of the methanol extracts from trifoliates of the subclover resistant varieties clearly showed a deterrence effect on feeding mites. As confirmation, pairwise choice tests with the extract from the susceptible variety Dalkeith also indicated a preference for the Dalkeith over those from the resistant varieties. Interestingly, the extracts obtained from the leaf surface of the resistant varieties were also found to be strong feeding deterrents.

TABLE 3. MEAN NUMBER OF MITES ON CONTROL AND TEST MEMBRANE SACHETS CONTAINING LEAF SURFACE EXTRACTS FROM DIFFERENT SUBCLOVER VARIETIES OVER 3 HOURS[a]

Concentration	Control (mean ± SE)	Treatment (mean ± SE)	Deterrence (%)	*P*
1%				
Dalkeith	4.05 ± 0.472	1.87 ± 0.506	36.8	<0.001
SE016	5.06 ± 0.578	0.67 ± 0.205	76.6	<0.001
SE020	6.41 ± 0.504	1.58 ± 0.220	60.4	<0.001
SE014	7.11 ± 1.271	0.60 ± 0.142	84.4	<0.001
0.5%				
Dalkeith	3.35 ± 0.280	5.40 ± 0.420	−23.4	0.002
SE016	5.14 ± 0.738	3.41 ± 0.441	20.2	NS
SE020	4.90 ± 0.482	3.18 ± 0.346	21.3	0.009
SE014	5.35 ± 0.859	3.50 ± 0.557	20.9	NS

[a]Both control and test membrane sachets contained 1% glucose and 5% Tween 80.

TABLE 4. AMOUNTS OF ISOFLAVONE DERIVATIVES IN METHANOL EXTRACTS FROM TRIFOLIATES OF VARIETIES OF SUBCLOVER

	Amount (μg/g fresh weight ± SE)				
Compound[a]	Dalkeith	SE016	SE020	SE011	SE014
Biochanin A	106 ± 7	288 ± 0.1	1791 ± 86	1538 ± 27	1648 ± 28
Genistein	325 ± 5	361 ± 1.3	761 ± 8	1327 ± 12	482 ± 5
Formononetin	22 ± 1.9	32 ± 0.8	170 ± 15	86 ± 8.5	164 ± 20
Subtotal	455	681	2722	2951	2294
BG	10 ± 0.7	41 ± 0.1	57 ± 7	1312 ± 20	178 ± 6
GG	8 ± 0.1	32 ± 0.2	43 ± 1.3	1312 ± 55	62 ± 6.2
FG	—	38 ± 0.4	47 ± 2.4	118 ± 17	48 ± 3.3
Subtotal	18	111	147	2732	288
BGM	—	94 ± 0.5	142 ± 7.9	—	114 ± 5
GGM	70 ± 4.6	89 ± 0.4	140 ± 8.1	35 ± 1.5	82 ± 7.2
Subtotal	70	183	282	35	196
Total	543	975	3151	5718	2778

[a]BG, biochanin 7-*O*-glucoside; GG, genistein 7-*O*-glucoside; FG, formononetin 7-*O*-glucoside; BGM, genistein 6″-malonyl-7-*O*-glucoside; GGM, genistein 6″-malonyl-7-*O*-glucoside.

TABLE 5. AMOUNTS OF ISOFLAVONE DERIVATIVES IN METHANOL EXTRACTS FROM TRIFOLIATE SURFACES OF VARIETIES OF SUBCLOVER

	Amount (μg/g fresh weight ± SE)				
Compound[a]	Dalkeith	SE016	SE020	SE011	SE014
Biochanin A	2.0 ± 0.13	1.1 ± 0.005	2.4 ± 0.20	6.8 ± 0.04	3.8 ± 0.05
Genistein	0.6 ± 0.004	2.2 ± 0.003	0.9 ± 0.06	7.7 ± 0.02	1.7 ± 0.01
Formononetin	—	0.2 ± 0.00	0.4 ± 0.03	1.0 ± 0.02	—
Subtotal	2.6	3.5	3.7	15.5	5.5
BG	—	4.2 ± 0.007	3.5 ± 0.01	7.6 ± 0.04	9.2 ± 0.02
GG	2.5 ± 0.05	8.1 ± 0.24	3.8 ± 0.23	7.6 ± 0.03	5.6 ± 0.02
FG	7.2 ± 0.28	15.0 ± 0.22	3.4 ± 0.001	0.8 ± 0.02	16.4 ± 0.23
Subtotal	9.7	27.3	10.7	16.0	33.2
BGM	1.1 ± 0.07	7.1 ± 0.08	2.5 ± 0.12	0.2 ± 0.003	29.7 ± 0.15
GGM	1.3 ± 0.12	4.4 ± 0.39	3.5 ± 0.22	0.3 ± 0.01	3.4 ± 0.05
Sub-total	2.4	11.5	6.0	0.5	33.1
Total	14.7	42.3	20.4	32.0	71.8

[a]BG, biochanin 7-*O*-glucoside; GG, genistein 7-*O*-glucoside; FG, formononetin 7-*O*-glucoside; BGM, genistein 6″-malonyl-7-*O*-glucoside; GGM, genistein 6″-malonyl-7-*O*-glucoside.

In a previous investigation of the components of the methanol extract of trifoliates of SE014, we identified the major extractable metabolites present (Wang et al., 1998a). The free isoflavones were represented by biochanin A, genistein, and formononetin and were accompanied by their corresponding 7-*O*-glucosides and the 6″-malonyl-7-*O*-glucosides of biochanin A and genistein. A number of other compounds were also identified as minor components, including the biosynthetic precursors of genistein and biochanin A. All these compounds showed deterrent activity towards feeding by mites, although the free isoflavones were considerably more active (0.05–0.15% concentrations) than the glycosylated isoflavones (0.5%) (Wang et al., 1998b).

The analyses of the types and amounts of these compounds in the methanol extracts of the five varieties showed that there were considerable differences between varieties. The Dalkeith variety contains much less of the compounds than any of the more resistant varieties, although these varieties differ among themselves. For example, SE016 has comparable levels of free isoflavones to those of Dalkeith, although it has markedly higher levels of the glycosylated isoflavones. While these results appear to indicate the significance of high levels of isoflavone derivatives in resistant varieties, they do not reflect the relative resistance of observed among these varieties under various conditions. Thus, by using criteria such as feeding damage scores in choice and non-choice tests, the most resistant variety is SE014 followed by SE011/SE020, SE016, and Dalkeith (Ridsdill-Smith, unpublished results; Gillespie, unpublished results). Such a sequence is not reflected by the total amounts of isoflavones found in the methanol extracts of the different varieties, or by the amounts of the three individual structural entities, free isoflavones, 7-*O*-glucosides, or the 6″-malonyl-7-*O*-glycosides.

A similar analysis of the components present on the leaf surface presents a better prediction of resistance. These results also show a marked difference in levels of isoflavones between Dalkeith and the resistant varieties. More significantly, the ranking of decreasing resistance of the varieties is similar to the ranking of decreasing levels of isoflavones SE014 > SE016 ≥ SE01 ≥ SE020 > Dalkeith. The fact that SE011 is relatively more resistant than SE016 may be due to the higher levels of free isoflavone on the leaf surface of SE011 than the other varieties. Deterrence data and estimates of the leaf components were collected for only four varieties, an insufficient number for multilinear regression analysis of the role of individual components. However, trends can be seen using ranking and simple correlation analysis. Of the individual components, the best prediction of deterrence was with 7-*O*-glucoside biochanin A (84% of variability in a linear regression). The subtotal of the glucosides gave the best prediction of deterrence (82% of variability of the subtotals), but this was only slightly greater than the total of all isoflavone derivatives (78% of variability), which is easier to measure. Total free isoflavones relatively gave a lower prediction of deterrence (69% of variability).

We interpret the results to mean that the amounts of total free isoflavone and their derivatives present on the leaf surface are major determinants in the resistance of a subclover variety to feeding by the redlegged earth mite. This is of some interest since we have observed glandular trichomes on the surface of these subclover varieties (Ridsdill-Smith, unpublished results) and the production of protective compounds by trichomes is a well-recognized phenomenon (Hashidoko, 1996).

A number of points need to be considered in the interpretation of the results. Instability during extraction of the bound isoflavones is known and may be responsible for an overestimation of the levels of free flavones (Beck, 1964). We attempted to minimize this effect by treating the leaf material with liquid nitrogen prior to extraction. This is less of a problem in the quantitation of the different isoflavone derivatives accumulating on the leaf surface, as enzymes are not released in the process of obtaining the metabolites.

Although we have previously shown that the isoflavone derivatives exhibit deterrence to mite feeding, our results do not exclude the possibility that the immediate deterrent factors are, in fact, compounds derived from these isoflavones. The formation of phytoalexins in leguminous plants from isoflavone precursors has been established (Barz and Welle, 1992). The greater deterrent activity of these phytoalexins compared to their precursor isoflavones towards some insect pests has been shown (Lane et al., 1985; Russell et al., 1979; Sutherland et al., 1980).

From a practical point of view, the results presented here suggest that the relative resistance of a subclover variety could be determined by measuring the levels of total isoflavone derivatives present on the leaf surface. Moreover, it appears that high levels of formononetin are not necessary for a variety to show resistance to redlegged earth mite. This is of some importance given the role of formononetin as a phytoestrogen which causes infertility in farm animals (Beck, 1964).

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TWO PHEROMONES OF CONIFEROPHAGOUS BARK BEETLES FOUND IN THE BARK OF NONHOST ANGIOSPERMS

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Abstract—Volatiles from fresh bark of black cottonwood, *Populus trichocarpa*; trembling aspen, *P. tremuloides*; paper birch, *Betula papyrifera*; bigleaf maple, *Acer macrophyllum*; red alder, *Alnus rubra*; and Sitka alder, *Alnus viridis*, were collected on Porapak Q and subjected to coupled gas chromatographic–electroantennographic detection analyses by utilizing the antennae of several scolytid beetles (*Dendroctonus pseudotsugae*, *D. rufipennis*, *D. ponderosae*, *Ips pini*, and *Dryocoetes confusus*). Among the antennally active volatiles identified by coupled gas chromatographic–mass spectroscopic analysis were frontalin, 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]-octane, in the two *Alnus* species and conophthorin, (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane, in the other four species. Field trapping experiments demonstrated that conophthorin had a significant disruptant effect on the response to a pheromone-host kairomone blend by both *Dendroctonus pseudotsugae* and *D. ponderosae*. Our results, and the recent identification of other scolytid pheromones in various tree species, pose major questions regarding the evolution and ecological roles of these semiochemicals, including the possibility of Batesian mimicry by the beetles. They also suggest a need for comparative studies on the biosynthetic pathways for these compounds.

Key Words—Semiochemicals, pheromones, nonhost volatiles, frontalin, conophthorin, 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane, Coleoptera, Scolytidae, Batesian mimicry.

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INTRODUCTION

Bark beetles (Coleoptera: Scolytidae) encounter several challenges upon emergence from their brood tree. First, they must find the few susceptible hosts widely scattered among a variety of nonhosts and resistant hosts (Rudinsky, 1962; Atkins, 1966). Second, they must minimize search time due to energy constraints (Bell, 1990). Third, searching beetles must avoid prolonged exposure to factors such as predation or inclement weather that threaten their fitness (Dahlsten, 1982; Gries et al., 1989). The requirement for high search efficiency will select for host-seeking beetles that utilize as much of the relevant information in the environment as is available to them.

Much research has focused on the powerful aggregation pheromones that act in synergy with host kairomones to mediate mass attack by conspecifics on new hosts (Borden, 1982). However, in the absence of aggregation pheromones, pioneer beetles must depend on other cues. These may be host kairomones (Byers, 1995) and visual stimuli (Shepherd, 1966) that allow the beetles to orient to appropriate hosts and nonhost kairomones that allow them to avoid inappropriate hosts (Dickens et al., 1992; Schroeder, 1992; Wilson et al., 1996; Borden et al., 1998; Deglow and Borden, 1998).

We have begun an intensive search into the identities and behavioral effects of volatiles found in the bark of nonhost angiosperms. Using the antennae of five species of bark beetles, the Douglas-fir beetle, *Dendroctonus pseudotsugae*; the mountain pine beetle, *D. ponderosae*; the spruce beetle, *D. rufipennis*; the pine engraver, *Ips pini*; and the western balsam bark beetle, *Dryocoetes confusus*, we have identified numerous antennally active compounds that emanate from the bark and phloem tissues of six species of nonhost angiosperms. The nonhost angiosperms in this study, black cottonwood, *Populus trichocarpa*; trembling aspen, *P. tremuloides*; paper birch, *Betula papyrifera*; bigleaf maple, *Acer macrophyllum*; red alder, *Alnus rubra*; and Sitka alder, *Alnus viridis*, are sympatric with most or all of the bark beetles tested (Borden et al., 1997).

Most of the compounds identified are common plant volatiles, e.g. benzyl alcohol, 1-hexanol, benzaldehyde, and nonanal (Borden et al., 1998), which have been shown or are hypothesized to be active in disruption of the response of searching bark beetles to attractant-baited traps. We report here the unexpected discovery of two scolytid pheromones, conophthorin, (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane, and frontalin, 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, in the bark of angiosperm trees.[3] Conophthorin is a male-produced antiaggregation pheromone in cone beetles, *Conophthorus* spp.; the ash bark beetle, *Leperisinus varius*; the fir bark beetle, *Cryphalus piceae*; and some twig beetles, *Pityophtho-*

[3] A preliminary account of this study was presented at the International Society of Chemical Ecology, 14th Annual Meeting, Vancouver, British Columbia, Canada, July 12–16, 1997, Abstract C-1-5.

rus spp. (Kohnle et al., 1992; Dallara et al., 1994; Pierce et al., 1995; Birgersson et al., 1995). It is an aggregation pheromone in some twig bettles and an attractant kairomone for the scolytid associates, *Epuraea thoracica* and *Lasconotus pertenius* (Kohnle et al., 1992; Dallara et al., 1994). It is also a possible aggression inhibitor in the wasp, *Paravespula vulgaris* (Francke et al., 1978). Frontalin is an aggregation pheromone in several *Dendroctonus* spp. (Borden, 1985) and is also a multifunctional pheromone for *D. ponderosae* (Ryker and Libbey, 1982; Borden et al., 1987).

METHODS AND MATERIALS

Black cottonwood and bigleaf maple were collected in February 1996 at the Malcolm Knapp Research Forest, University of British Columbia, Maple Ridge, British Columbia. Sitka alder was collected in February 1996 near Princeton, British Columbia. Red alder was collected in January 1996 in Burnaby, British Columbia. Paper birch and trembling aspen were collected in March 1996 near Boston Bar, British Columbia. Felled trees were cut into bolts that were either used immediately or stored at −12°C for no more than seven days.

For each species, bark was stripped from the bolts, cut into pieces (ca. 9 cm^2), and placed in chambers (ca. 1 kg of bark in a 10-liter chamber). Air was drawn through the chamber for three to four days at ca. 23°C with an airflow maintained near 2.5 liters/min. Volatiles were captured on Porapak Q and recovered by extraction with pentane (Pierce et al., 1981).

Beetle-infested material was collected as follows: Douglas-fir beetles in bark, March 1996 near Boston Bar, British Columbia; spruce beetles and western balsam bark beetles in infested bolts, August 1996 near Coalmont, British Columbia; and mountain pine beetles and pine engravers in infested bolts, November 1995 and September 1996, respectively, both near Princeton, British Columbia. Collected bolts were waxed on both ends. Infested material collected in the late summer or fall was overwintered outside. Infested bolts and bark were placed in cages at 27°C and emerged beetles were collected daily.

Coupled gas chromatographic–electroantennographic detection analysis (GC-EAD) was carried out on live beetles of the first-attacking sex (female *Dendroctonus* spp., male *Ips pini* and *Dryoceotes confusus*) with a HP 5890 gas chromatograph containing a fused silica column (DB-5, 30 m × 0.25 mm ID, J&W Scientific, Folsom, California, 95630-4714) as described by Gries (1995). Antennally active compounds were identified by cochromatography with authentic samples and by coupled gas chromatographic–mass spectroscopic analysis (GC-MS). Before chiral analysis, frontalin and conophthorin were isolated by high-performance liquid chromatography (HPLC) by employing a Waters LC 626 system, a Waters 746 data module, and a normal phase column (Nova-

Pak Silica, 3.9 mm × 150 mm HPLC cartridge column) with 1 ml/min isocratic flow of 5% ether–95% hexane for frontalin and 3% ether–97% hexane for conophthorin. Enantiomeric composition of conophthorin and frontalin was determined on a Cyclodex B column (30 m × 0.25 mm ID, J&W Scientific). Conophthorin from paper birch, cottonwood, and trembling aspen were chromatographed isothermally at 85°C on the chiral column under split conditions. Conophthorin from broadleaf maple was chromatographed on the chiral column under splitless conditions (80°C, hold for 2 min, then increase temperature 2°C/min to 100°C). Frontalin from both alder species was chromatographed on the chiral column under splitless conditions (80°C, hold for 1 min, then increase temperature 1°C/min to 95°C).

Because it is a nonhost volatile and because it generally elicits negative responses in a variety of taxa, synthetic conophthorin (Pierce et al., 1995) was tested in the field for its ability to disrupt response to 12-unit, multiple-funnel traps (Lindgren, 1983) baited with attractants for *D. pseudotsugae* and *D. ponderosae* (Table 1). Traps were placed in lines with each trap at least 15 m from adjacent traps in randomized complete blocks. A small block of Vapona No-Pest Strip (Green Cross, Fisons Horticulture Inc. 600-25 Watline Avenue, Mississauga, Ontario L4Z

TABLE 1. SUMMARY OF COMPOUNDS USED IN FIELD-TRAPPING EXPERIMENTS

Chemical	Release device	Source	Release rate (mg/24 hr)
Conophthorin	closed 0.5-ml Eppendorf tube	Simon Fraser University	0.5[b]
D. pseudotsugae bait			
Ethanol	40-cm PVC pouch	Phero Tech[a]	40[c]
Frontalin	400-μl vial	Phero Tech	2.6[d]
MCOL	bubble cap	Phero Tech	2.0[c]
D. ponderosae bait			
exo-Brevicomin	flex lure	Phero Tech	0.28[c]
Myrcene	15 ml bottle	Phero Tech	95[d]
trans-Verbenol	bubble cap	Phero Tech	1.5[c]

[a]7572 Progress Way, Delta, British Columbia, V4G 1E9, Canada.
[b]Release rate determined at Simon Fraser University, 27°C.
[c]Release rate determined at Phero Tech, 20°C.
[d]Release rate determined at Phero Tech, 23°C.

2Z1) was placed in each collecting cup so that predaceous beetles did not alter trap catches. Experiment 1 was designed to test the repellent effect of conophthorin against the Douglas-fir beetle with three treatments: attractant alone or combined with conophthorin and an unbaited control. Experiment 2 was a test of conophthorin alone and the attractant alone for the Douglas-fir beetle. Experiment 3 was a test of the effect of conophthorin on mountain pine beetle with four treatments: attractant alone or with conophthorin, conophthorin alone, and an unbaited control. Dates and locations were as follows: experiments 1 and 2, May 15–20 and June 10–13, 1997, respectively, near Lytton, British Columbia, and experiment 3, July 18–23, 1997, near Princeton, British Columbia. Captured beetles were collected and stored at −12°C until they were sexed and counted.

Trap catches were converted to the mean proportions of each sex within a replicate, transformed by arcsine of the square root (Zar, 1984), and analyzed with SAS Institute Inc. software (GLM procedure, SAS Institute Inc., 1988) by ANOVA and the Ryan-Einot-Gabriel-Welsch (REGW) multiple-Q test (Day and Quinn, 1989).

RESULTS

Frontalin was detected by GC-EAD in the bark volatiles of *Alnus rubra* and *A. viridis*, and conophthorin was found in the bark volatiles of *Betula papyrifera*, *Acer macrophyllum*, *Populus trichocarpa*, and *P. tremuloides* (Figure 1, Table

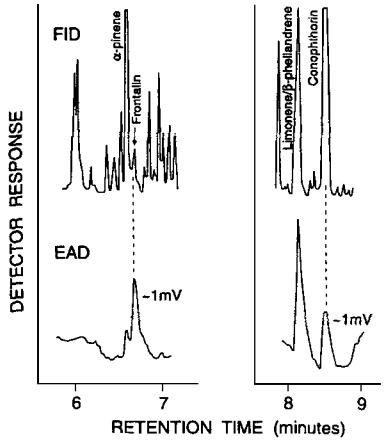

FIG. 1. Responses of female Douglas-fir beetle antennae to frontalin in red alder (left) and to conophthorin in paper birch (right). Upper traces are portions of the flame ionization detector responses to volatile compounds in the two bark aeration extracts; lower traces are electroantennogram detection responses to the compounds in the aeration extracts.

TABLE 2. CONTENT OF FRONTALIN AND CONOPHTHORIN IN CAPTURED BARK VOLATILES OF SIX ANGIOSPERM TREE SPECIES

Nonhost angiosperm species	Percent of total bark volatiles[a]	
	Frontalin	Conophthorin
Red alder	0.090	ND
Sitka alder	0.074	ND
Paper birch	ND	12.086
Black cottonwood	ND	1.098
Trembling aspen	ND	0.482
Bigleaf maple	ND	0.437

[a]ND = not detected.

2). Conophthorin has also recently been found in the bark of *Betula pendula* and *B. pubescens* in Sweden (Byers et al., 1998). In all but one case in the trees that we surveyed these two scolytid pheromones were minor constituents of the bark volatiles. However, conophthorin was a major constituent (>12%) of the bark volatiles in *B. papyrifera*. (+)- and (−)-Frontalin were found in a 1 : 4 ratio, while (+)- and (−)-conophthorin were found in a 1 : 9 ratio. There was little variation between tree species in enantiomeric composition.

Conophthorin in bark volatiles elicited an antennal response from all five scolytid species. Antennae of the three *Dendroctonus* spp. responded to frontalin, but *Ips pini* and *Dryocoetes confusus* did not. Because frontalin did not excite the antennae of *I. pini* or *Dryocoetes confusus*, and because its bioactivity on *Dendroctonus* spp. is well known, no field tests of this compound were carried out. Conophthorin released at about 0.5 mg/24 hr disrupted the response of male and female *D. pseudotsugae* to attractant-baited traps by 32% and 35%, respectively (Table 3). For *D. ponderosae* the respective rates of disruption were 50% and 32%. Conophthorin alone did not attract beetles of either species.

DISCUSSION

Blum's (1970, 1996) "law" of pheromonal parsimony among insects is widely accepted. Our results, and those of others, indicate that this law should be expanded into an overall "Rule of Semiochemical Parsimony." That is, certain chemicals, because of their low biosynthetic cost or because they possess certain physical qualities, e.g., volatility, have been adopted by disparate organisms for use in diverse communicative functions. For instance, 1-octen-3-ol is a kairomone found in cattle odor that attracts the tsetse flies, *Glossina pallidipes* and *G. morsitans morsitans* (Hall et al., 1984), and is also used as an aggregation

TABLE 3. CAPTURES OF *Dendroctonus pseudotsugae* AND *D. ponderosae* IN MULTIPLE-FUNNEL TRAPS IN THREE FIELD-TRAPPING EXPERIMENTS TESTING BIOACTIVITY OF CONOPHTHORIN ON RESPONSES TO ATTRACTIVE BAITS

Expt.	Treatment	*N*	Percent of total captured (mean ± SE)[a] Males	Females
1	DFB bait	11	59.2 ± 4.1 a	59.0 ± 5.1 a
	DFB bait + Conophthorin	11	40.4 ± 4.2 b	38.6 ± 4.7 b
	Unbaited	11	0.5 ± 0.3 c	2.5 ± 1.2 c
2	DFB bait	8	99.4 ± 0.3 a	97.6 ± 0.8 a
	Conophthorin	8	0.3 ± 0.2 b	0.9 ± 0.5 b
	Unbaited	8	0.3 ± 0.2 b	1.5 ± 0.4 b
3	MPB bait	10	66.9 ± 3.2 a	59.4 ± 4.9 a
	MPB bait + Conophthorin	10	33.1 ± 3.2 b	40.6 ± 4.9 b
	Conophthorin	10	0.0 ± 0.0 c	0.0 ± 0.0 c
	Unbaited	10	0.0 ± 0.0 c	0.0 ± 0.0 c

[a]Means within an experiment and sex followed by the same letter are not significantly different, REGW test, $P > 0.05$.

pheromone by the grain beetles, *Oryzaephillus surinamensis* and *O. mercator* (Pierce et al., 1989). The widely occurring lepidopteran sex pheromone (*Z*)-7-dodecen-1-yl acetate (Arn et al., 1992) is also utilized as a sex pheromone by female Asian elephants, *Elephas maximus* (Rasmussen et al., 1996). Frontalin has also been recently identified as a component of temporal gland secretion in male *E. maximus* and may possibly elicit an avoidance response in female elephants (Perrin et al., 1996). Benzaldehyde has been identified as a male hair-pencil pheromone component in some noctuid moths (Fitzpatrick et al., 1985; Jacquin et al., 1991), as an aggregation pheromone component in the desert locust, *Schistocerca gragaria* (Torto et al., 1994), and as a repellent angiosperm bark volatile for the mountain pine beetle (Borden et al., 1998).

Several examples have been noted recently of plants and scolytid beetles producing identical volatile compounds. Verbenone, 4,6,6-trimethylbicyclo-[3.1.1]hept-3-en-2-one, a common antiaggregation pheromone used by several *Dendroctonus* spp. (Borden, 1985), has been identified from twigs of eastern cottonwood, *Populus deltoides* (Carlos Flechtmann, Departamento de Biologia, FEIS/UNESP, Av. Brasil, 56, 15 385-000-Ilha Solteira-SP, Brazil, personal communication), a tree species with a range overlapping that of the southern pine beetle, *Dendroctonus frontalis*. 2-Methyl-3-buten-2-ol (MBO) has been identified in foliar emissions of several North American *Pinus* spp. at levels that could result in concentrations of about 1.5 ppb of the chemical in a stand (Harley et al.,

1998). Several European spruce-infesting scolytids, including *Ips typographus* (Bakke et al., 1977; Schlyter et al., 1987; Birgersson, 1989) and *Ips amitinus* (Zuber, 1994), use MBO as a pheromone, and it is disruptive to *Dendroctonus rufipennis* in North America (Werner and Holsten, 1995). Although in the case of MBO many of the beetles and the trees are allopatric, it is possible that our understanding of *Ips* spp. pheromones is incomplete and that we will yet find MBO in the volatiles emitted by some North American *Ips* spp. In the above examples, as well as that of frontalin and conophthorin in the bark of several angiosperm trees, the trees that produce the chemicals given in the examples are nonhosts for the bark beetles that use the chemical as a pheromone. We have several reasons to postulate that for bark beetles these examples represent more than simple parsimony.

First, in many of the examples of parsimony noted above, e.g., compounds shared by elephants and insects, none of the species that utilize the same chemical actually interact and, in fact, many are allopatric. On the other hand, the bark beetles and the nonhost angiosperm trees that contain conophthorin, frontalin, and verbenone certainly must interact in a mixed forest environment. Second, scolytid beetles do indeed sense and react negatively to the presence of conophthorin, frontalin, and verbenone (Ryker and Libbey, 1982; Borden, 1985; Borden et al., 1987; Kohnle et al., 1992; Dallara et al., 1994; Birgersson et al., 1995; Pierce et al., 1995). Third, there are metabolic costs for both the beetles and the trees to synthesize semiochemicals, suggesting an adaptive advantage for both to produce identical compounds.

A possible adaptive advantage to the trees is that conophthorin, frontalin, and verbenone may be odors that warn phytophagous insects of potential fitness consequences involved in consuming plant tissues. As occurs for the aposematic warning colors red and orange, these chemicals may represent general warning odors, a role previously postulated for alkylmethoxypyrazines (Guilford et al., 1987; Aldrich et al., 1996).

If conophthorin, frontalin, and verbenone represent general warning odors, it may be that various coniferophagous scolytids began to produce them as mimics of angiosperm trees in order to protect their host resource from other conifer-infesting insects. This would be a case of chemical Batesian mimicry (Bates, 1862) with the models being angiosperm trees and the mimics being scolytid beetles. Some bark beetles may then have secondarily evolved the capacity to use the chemicals as aggregation or antiaggregation pheromones; the mimicry of angiosperms would, however, be maintained as protection against other coniferophagous insects. Bark beetles using nonhost odors as aggregation pheromones in combination with other pheromones and host-produced kairomones would still be able to recognize nonhost angiosperm trees through the perception of other nonhost volatiles (Dickens, et al., 1992; Wilson et al., 1996; Borden et al., 1998; Deglow and Borden, 1998).

Whether or not they were acting as mimics, coniferophagous bark beetles releasing compounds as pheromones that were identical to some of those produced by the trees around them might have had an adaptive advantage of lacking apparency to potential predators that were searching in an angiosperm-rich environment already quite saturated with the same chemicals. Predators in turn would have adapted to respond to other pheromone and tree volatile cues (Dahlsten, 1982).

The presence of scolytid pheromones in the abundant resource of angiosperm tree bark may facilitate elucidation of the biosynthetic pathways of these compounds. It would also be of interest to determine whether the biosynthetic pathways in beetles and trees are divergent or convergent.

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LIGNOCERIC ACID AND HEXACOSANOIC ACID: MAJOR COMPONENTS OF SOLDIER FRONTAL GLAND SECRETIONS OF THE FORMOSAN SUBTERRANEAN TERMITE (*Coptotermes formosanus*)

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Abstract—Qualitative and quantitative analyses were performed on trimethylsilyl derivatives of soldier frontal gland secretions of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, by using gas chromatography–mass spectrometry. Ranges of 17.23–33.78 mg/g (acid/wet weight of secretion) of lignoceric acid and 5.39–7.67 mg/g of hexacosanoic acid were found in the soldier frontal gland secretion of three different colonies. This is the first report that organic acids occur in the soldier secretions of this species.

Key Words—Formosan subterranean termite, *Coptotermes formosanus*, soldier defense secretion, lignoceric acid, hexacosanoic acid.

INTRODUCTION

Soldiers of many termite species, including those of Formosan subterranean termites, *Coptotermes formosanus* Shiraki, are morphologically and physiologically specialized for defense (Quennedey and Deligne, 1975). In addition to their enlarged and heavily sclerotized mandibles, which are used to bite, pierce, shear, and cut intruders, Formosan subterranean termite soldiers are also armed with

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chemical weaponry. When disturbed, the soldiers secrete a gluelike white fluid from the frontal gland, which often forms a drop between the open mandibles. Although the frontal gland is generally assumed to be the source of defensive chemicals of termites (Clément and Bagnères, 1998), biological data are not available for many species (Prestwich, 1984). Waller and LaFage (1987) reported that the soldier secretion of *C. formosanus* did not deter ant attack but that soldier unpalatability is a passive defense strategy of this species. This conclusion is based on their observation that soldiers and workers were killed at the same rate in laboratory arenas when they were encountered by ants and that ants removed workers, but not soldiers, from the foraging arena. What causes the soldier unpalatability is unknown.

Soldiers of many termite species possess frontal glands (Deligne et al., 1981; Noirot, 1969). Chemical analyses have revealed an impressive diversity of chemical components in this gland: monoterpenes, diterpenes, sesquiterpenes, quinones, macrocyclic lactones, alkanes, alkenes, nitroalkanes, vinyl ketones, and ketoaldehydes. (Prestwich, 1979, 1983, 1984; Prestwich and Collins, 1982; Prestwich et al., 1977; Goh et al., 1990; Chuah et al., 1983; Spaton and Prestwich, 1981). In *Coptotermes*, the chemicals in the frontal gland vary among species. A heterogeneous suspension of *n*-alkanes and mucopolysaccharides was found in *C. lacteus* (Froggatt) (Moore, 1969). Hexadecanal and heptadecanal were found in *C. testaceous* (Linnaeus) (Blum et al., 1982). In the case of the Formosan subterranean termite, the chemical analysis of the soldier frontal gland has not been fully described, although Prestwich (1984) mentions that hydrocarbons and mucopolysaccharides are the major components.

As part of our recent attempt to analyze polar compounds in soldier frontal gland secretion of the Formosan subterranean termite, we report here that lignoceric acid and hexacosanoic acid are two major components of the frontal gland secretions of this species.

METHODS AND MATERIALS

Termites. Three different colonies were used in this experiment. Colonies (carton nest with termites) were collected from infested houses in New Orleans (two colonies) and Lake Charles (one colony), Louisiana. The collected colonies were placed in plastic trash cans and maintained at room temperature (23–28°C). Pine wood and cardboard rolls were provided as food. All colonies were kept in the laboratory for less than six months.

Preparation of Gland Secretion. Forty soldiers from each colony were used. When holding the termite soldier abdomen, each soldier was stimulated to secrete the contents of its frontal gland and form a white liquid drop between its mandibles. Clean fine forceps were used to collect the drop. The secretion

from each soldier was transferred to a 0.1-ml chromatograph vial (VWR Scientifics, Philadelphia, Pennsylvania). Forty microliters of tricosanoic acid (134 μg/ml) was added into the reaction vial as an internal standard. The sample was dried under N_2.

Derivatization of Gland Secretion. To detect the polar compounds, 40 μl trimethylsilylating agent, *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (Pierce, Rockford, Illinois), were added to the reaction vial and the cap was immediately secured. The vial was vortexed for 20 sec., then heated at 70°C for 1 hr before the gas chromatography–mass spectrometry (GC-MS) analysis.

Instrumental Methods. Samples were analyzed on a Hewlett Packard 5890 gas chromatograph with a 5971A mass selective detector. The system was controlled by the Hewlett Packard Series 300 computer with HP 5990C Chemstation. The GC was equipped with a SGE BPX5 column (5% phenyl polysiloxane, 25 m long, 0.22 ID, film thickness 0.25 μm). Helium was used as a carrier gas with a linear flow velocity of 26.6 cm/sec. The initial temperature was 40°C for 3 min and increased at a rate of 20°C/min, until reaching a final temperature of 280°C. This final temperature was held for 25 min.

Identification and Quantification of Lignoceric and Hexacosanoic Acid. Identification of lignoceric acid and hexacosanoic acid was made by first searching the spectra library, then comparing the mass spectra and GC retention times of peaks in the sample with those of trimethylsilyl (TMS) derivatives of lignoceric acid and hexacosanoic acid standards (99+%, Sigma Chemical Co., St. Louis, Missouri). The calculation of acid concentrations was based on the relative peak area of lignoceric TMS (lignoceric acid trimethylsilyl ester) and hexacosanoic TMS, compared to the internal standard tricosanoic TMS.

RESULTS

Lignoceric acids and hexacosanoic acid were found in the samples from all three colonies. A total ion chromatogram of the trimethylsilyl derivatives from soldier frontal gland secretion is shown in Figure 1A. The retention time of lignoceric TMS was 20.81 min, that of hexacosanoic TMS, 24.36 min. The total ion chromatograms of trimethylsilyl derivatives of lignoceric acid and hexacosanoic acid, with the internal standard tricosanoic acid, are shown in Figure 1B and C. The mass spectra of the peaks at the GC retention times 20.81 and 24.36 min and those of standard lignoceric TMS and hexacosanoic TMS are shown in Figures 2A and 3A and Figures 2B and 3B, respectively. Identification was confirmed by comparing the retention times and mass spectra of both peaks at 20.81 and 24.36 min in samples with those of the standards. The mean concentration of lignoceric acid in the frontal gland secretion ranged from 12.22 to 33.78 mg/g and hexacosanoic acid from 5.39 to 7.67 mg/g for soldiers (Figure 4). The ratio

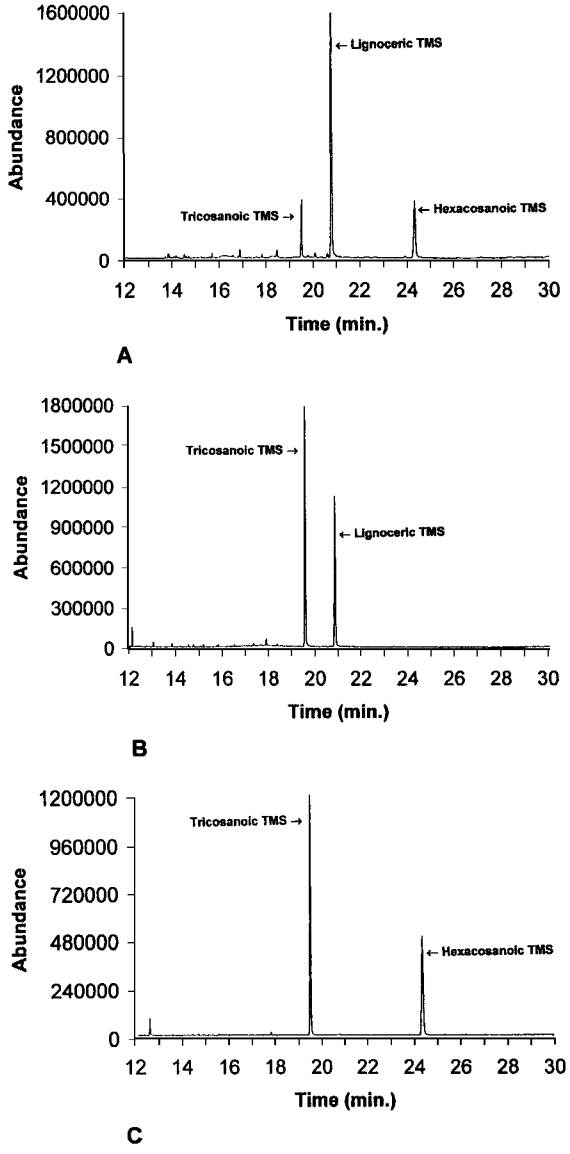

FIG. 1. Total ion chromatogram of sample and acid standards. (A) Total ion chromatogram of trimethylsilyl derivatives of Formosan subterranean termite soldier frontal gland secretion. (B) Total ion chromatogram of lignoceric TMS and internal standard tricosanoic TMS. (C) Total ion chromatogram of hexacosanoic TMS and internal standard tricosanoic TMS.

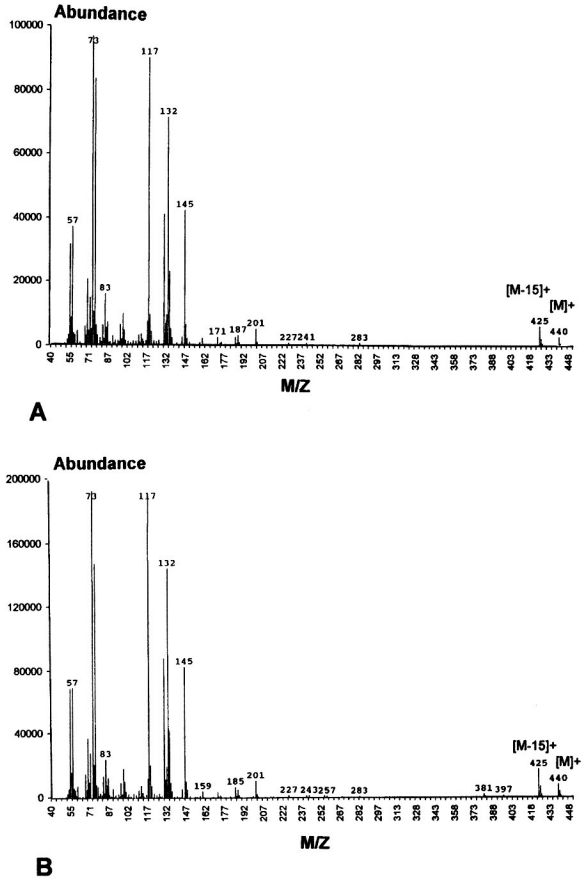

FIG. 2. Mass spectra of targeted peak and lignoceric acid standard. (A) Mass spectrum of the peak at GC retention time 20.81 min of soldier frontal gland secretion. (B) Mass spectrum of lignoceric TMS.

between lignoceric acid and hexacosanoic acid (pooled data) was 3.50 ± 0.69 (mean ± standard deviation).

DISCUSSION

Mucopolysaccharides and hydrocarbons have been reported as components of the Formosan subterranean termite soldier frontal gland secretion (Prestwich, 1984). The chromatographic conditions used in this experiment were such that

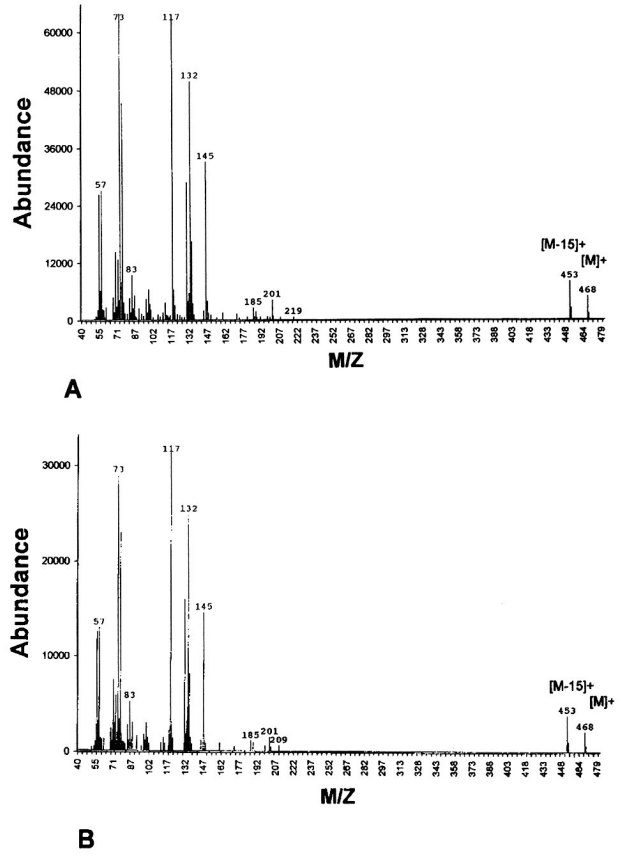

FIG. 3. Mass spectra of targeted peak and the hexacosanoic acid standard. (A) Mass spectrum of the peak at GC retention time 24.36 min of soldier frontal gland secretion. (B) Mass spectrum of hexacosanoic TMS.

long-chain hydrocarbons with fewer than 26 carbons, if present, should have been detected. However, no such compounds were detected. It has been mentioned that the hydrocarbons in Formosan subterranean termite soldier frontal gland secretion are derived from fatty acids (Prestwich, 1984), but no reference is available concerning the details of Formosan subterranean termite frontal gland chemistry.

It is believed that predation by ants determined the evolution of defensive traits of termite soldiers (Deligne et al., 1981; Mill, 1983). Howard et al. (1982) suggested that the use of fatty acids as an ant repellent is a widespread phenomenon among insects. Blum et al. (1982) reported that nonadecanoic acid

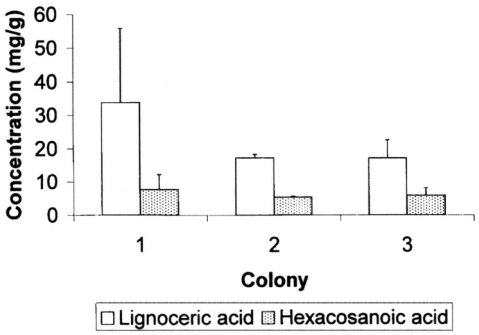

FIG. 4. Concentrations (means ± standard deviation) of lignoceric acid and hexacosanoic acid in frontal gland secretion of Formosan subterranean termite soldiers (based on wet weight of the secretion).

is the major component in the secretion of *Rhinotermes marginalis*. Prestwich (1984) stated that most termite soldiers with slashing or mandibles produce irritants such as volatile C_{12}–C_{20} fatty acids and terpenoid compounds. However, free fatty acids have not been previously reported in the soldier frontal gland secretion of Formosan subterranean termites.

In an experiment which evaluated the interaction between *C. formosanus* and three ant species, Waller and LaFage (1987) found that the soldier secretion did not forestall attack by ants but that the unpalatability of soldiers constituted a passive defense strategy. Frontal gland secretion accounts for up to 36% of the soldier fresh weight of *C. formosanus* (Waller and LaFage, 1987). Whether lignoceric acid and hexacosanoic acid contribute to soldier unpalatability remains to be verified, but the lack of deterrence of attacks by ants makes it unlikely that these long-chain fatty acids have the same irritant quality as those volatile fatty acids reported by Prestwich (1984). Finally, long-chain hydrocarbons are assumed to be an anti-healing component (Prestwich, 1979; Prestwich et al., 1977), which is another possible function of the long chain-fatty acids.

The chemicals from the soldier frontal gland can also serve as common nest signature and alarm pheromones (Clément and Bagnères, 1998). Whether lignoceric acid and hexacosanoic acid play any role in those aspects also remains to be determined.

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EVERSIBLE PHEROMONE GLAND IN A MELOLONTHINE BEETLE, *Holotrichia parallela*

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Abstract—The morphology of an eversible pheromone gland of a melolonthine beetle, *Holotrichia parallela* (Coleoptera: Scarabaeidae: Melolonthinae), was characterized. Through careful dissection of the gland and GC-MS analysis of tissue extracts, we determined that the pheromone is produced in the posterior part of a ball-shaped sac exposed during female calling. Light microscope observation of the posterior part of the gland revealed a layer of cuticular epithelium composed of columnar cells, which was assigned as the tissue involved in pheromone production. Other morphological features, such as a soft cuticular layer adjacent to the epithelium and groups of retractor muscles attached to the gland, were characterized according to their functions. Paired accessory glands, which in some other melolonthine species house symbiotic bacteria that produce a sex attractant, were found not to be involved in pheromone production in *H. parallela*.

Key Words—Pheromone gland, *Holotrichia parallela*, Coleoptera, Scarabaeidae, Melolonthinae, columnar cells, accessory gland.

INTRODUCTION

In beetles (Coleoptera), little is known about the morphology of sex-pheromone-producing glands, and studies are limited to the species in which the morphology of the glands provides a certain taxonomic significance (Plout-Sigwalt, 1982, 1986; Coca-Abia and Martin-Piera, 1991) or those that have unique histological features (Stringer, 1988). In a few genera of Scarabaeidae, including the genus *Anomala*, Zamotailov (1988) examined female reproductive structures and called paired lobelike accessory glands "pheromone glands." However, Tada and Leal

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(1997) demonstrated that the pheromone gland in *Anomala* spp. (subfamily Rutelinae) is, in fact, a group of integumental epithelial tissues on a few last abdominal segments; pheromone is emitted to the exterior through cuticular canals. Subsequent investigations revealed that this epithelial pheromone gland is a common feature among anomalan beetles but is not necessarily shared by species in other genera or closely related subfamilies.

In Melolonthinae, pheromone gland morphology seems more diverse than that of Rutelinae. For example, while *Heptophylla picea* has a simple anomalan-type pheromone gland (Tada and Leal, 1997), another melolonthine, *Costelytra zealandica*, harbors, in paired accessory glands, a symbiotic bacterium that produces an attractant to male beetles (Hoyt et al., 1971; Stringer, 1988). In yet another melolonthine, *Holotrichia parallela* (Motschulsky), Leal et al. (1993) observed that a calling female everted a ball-like structure from her abdominal tip, which contained high titers of the major pheromone component, L-isoleucine methyl ester (LIME) (Leal et al., 1992). Although a similar ball-like structure is seen in other melolonthine beetles (Coca-Abia and Martin-Piera, 1991), its detailed morphology and function with respect to pheromone production and emission have not yet been studied. In order to provide a basis for future studies on the biosynthesis of melolonthine sex pheromones, we have examined the morphology of the eversible gland of *H. parallela* and determined the localization of pheromone-producing tissues.

METHODS AND MATERIALS

Dissection of Pheromone Glands. H. parallela females were collected from two different areas in Tsukuba, Ibaraki Prefecture. Since females emit the pheromone every other day (Leal et al., 1993), they were collected on emission days and dissected immediately after collection. By squeezing the abdomen of anesthetized females, a ball-like structure housing a pheromone-producing gland was everted and was removed by scissors under a dissection microscope. The ball-like structure was further dissected into four parts: anterior part (AN), middle part (MI), posterior part (PO), and paired accessory glands (AG) (Figure 1A). To obtain tissue extracts, these four parts were individually washed with 50 μl of dichloromethane; methyl octanoate was added as an internal standard (50 ng/μl). Seven replicates (seven females) were made for each dissected part.

GC-MS Analyses. GC analyses of tissue extracts were performed on an HP 6890 gas chromatograph linked to a mass selective detector MSD 5973 (Hewlett-Packard). Chromatographic resolution was achieved on an HP-5MS column (30 mm $\times$ 0.22 mm $\times$ 0.25 μm: Hewlett-Packard). The column was operated at 50°C for 1 min, temperature increased to 100°C at a rate of 5°C/min, and then increased to 250°C at a rate of 10°C/min.

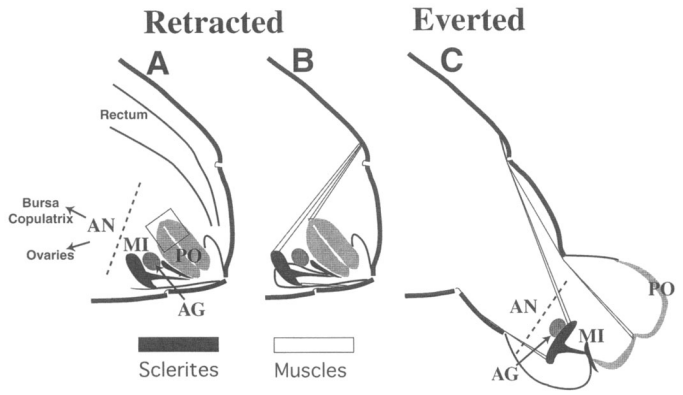

FIG. 1. A schematic diagram of a longitudinal section of the last two abdominal segments in female *H. parallela*. (A) The eversible structure composed of anterior part (AN), middle part (MI), posterior part (PO) (=pheromone gland) and paired accessory glands (AG). A dotted line indicates the incision separating AN and MI, and an open square approximates the tissue section observed by the light microscope (see Figure 2D). (B) A side view of PO, associated muscles, and sclerites in the retracted position. (C) A side view of the everted position.

Tissue Dissection and Preparation for Microscopic Observation. Adult females were fixed for 1 hr by injection with 70% ethanol. The ventral surface of the last two abdominal segments was opened to expose the pheromone gland. Ovaries, bursa copulatrix, and rectum were removed. Muscle attachments to external and internal sclerites were carefully examined under a dissection microscope.

For light microscope observation, the whole ball-like structure with the pheromone gland inside was fixed in Bouin's fixative for two days. After dehydrating with graded series of ethanol and toluene, it was embedded in molten paraffin. Thin-sectioned specimens were stained with Harris hematoxylin, counterstained with eosin yellow, and observed under an Olympus M081 transmission light microscope.

RESULTS

When the female's abdomen was squeezed with fingers, a ball-like structure was everted with a distance of about twice the longitudinal length of the pygidium. On being pushed out from the abdominal tip, the posterior part of the structure (PO) emerged, exposing dorsal and ventral lips (Figure 2B). As the abdomen was further squeezed, these lips protruded, opened up like a hinge,

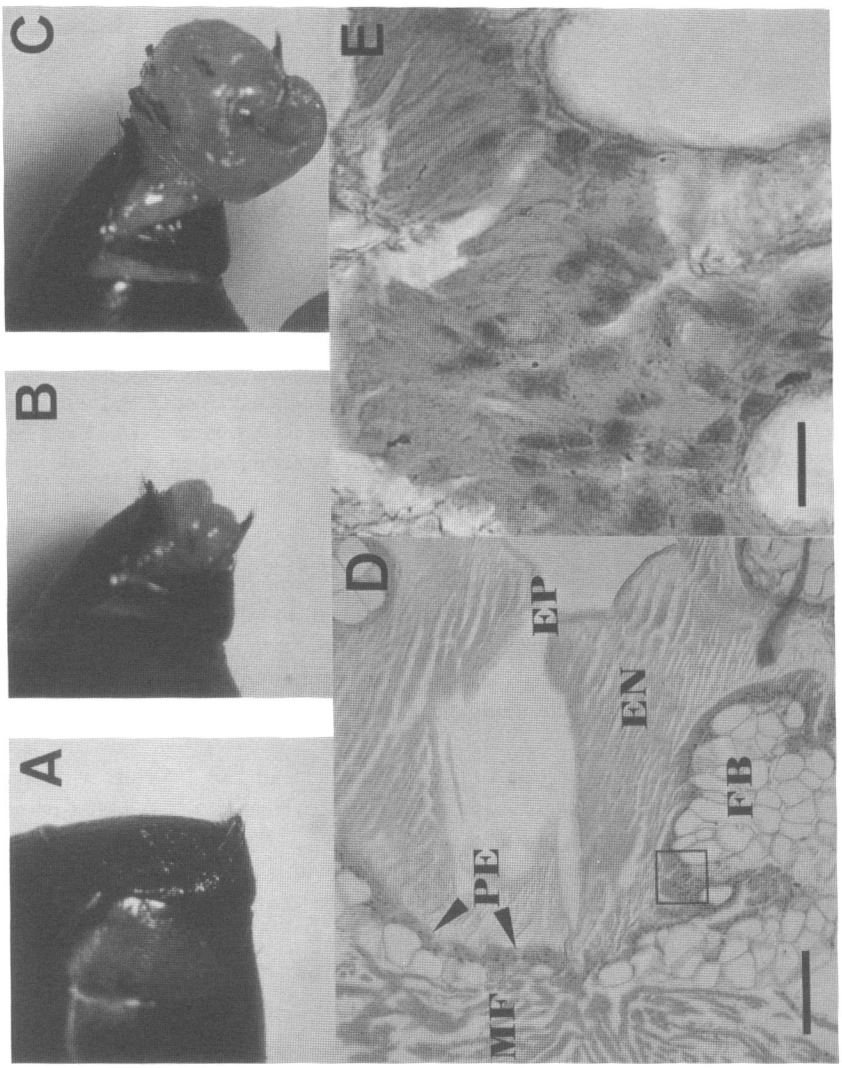
A
B
C
D
E
MF
PE
EP
EN
FB

FIG. 2. Side views of a female abdominal tip. The tip of the abdomen is closed and the pheromone gland is completely hidden in the abdomen (A). The tip slightly opened to allow dorsal and ventral lips of the pheromone gland to emerge (B); these lips opened up, flipped over, and fully expanded at the everted position (C). Longitudinal sections of the pheromone gland showing the pheromone-producing tissue and surrounding tissues (D): from left to right, muscle fibers (MF), pheromone-producing epithelium (PE), clusters of fat body (FB), a thick soft endocuticle (EN), and a thin epicuticle (EP); bar = 200 μm. (E) A magnified view of the pheromone-producing epithelium (the section marked by a square in D) showing clusters of columnar cells; bar = 20 μm.

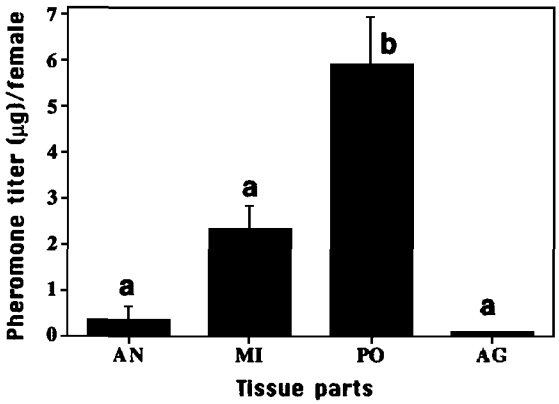

FIG. 3. Mean titers of LIME extracted from tissue parts. Acronyms are identical to those in Figure 1. Values with different letters are significantly different from each other by Scheffe *F* test at the 5% level. Error bars indicate standard errors.

flipped over, and expanded to form a wide cuticular surface (Figure 2C). When the squeezing was stopped, the cuticular surface shrank in size, the two lips closed, and the entire structure was pulled into the abdomen (Figure 2A).

Examination of the various tissue extracts by GC-MS showed that extracts from the posterior part of the ball-like structure (PO) contained the highest titer of LIME, the major component of the pheromone (Figure 3), indicating the occurrence of pheromone-producing glands. The anterior (AN) and the middle parts (MI) of the everted structure had significantly lower titers. The paired accessory glands located above the internal sclerite had a significantly lower pheromone titer than the other parts (Figures 1A and 3).

Observations of the paraffin sections of the pheromone gland (PO) under the light microscope revealed an exocrine tissue. The tissue was composed of a relatively thin layer of cuticular epithelium immediately exterior to the fat body (Figure 2D). The epithelial layer formed a U-shaped arrangement, which corresponded to the folded section of dorsal and ventral lips (Figure 1A). These epithelial cells were more or less evenly distributed along the entire tissue but formed some irregular clusters merging into the fat body (Figure 2D). Under a higher magnification, these cells appeared columnar-shaped (Figure 2E), and within each cell a nucleus was located close to the basal end. A thick and soft endocuticle protecting the underlying tissues was located exterior to these cells (Figure 2D).

Three groups of retractor muscles were involved in the movement of the pheromone-producing gland (Figure 1B). One of them was attached at the center of the folded section of the posterior part of the structure (PO). This muscle

extended beyond the tip of the abdomen when the whole structure was fully everted (Figure 1C). The other two groups of muscles behaved in a similar way but were involved in the retraction of associated internal sclerites. During eversion, these sclerites supported the shape and movement for the rest of the everted structure composed of anterior (AN) and middle (MI) parts and paired accessory glands (AG).

DISCUSSION

The abdominal eversible structure in *H. parallela* housed the pheromone gland in the posterior end, which was exposed and engorged when the abdomen was squeezed. This eversible pheromone gland functionally resembles some lepidopteran counterparts (Percy-Cunningham and Weatherston, 1974; Percy-Cunningham and MacDonald, 1987), where associated structures not only protect the more delicate tissue parts from injury but also help increase the efficiency of pheromone emission during calling. However, the exact mechanisms of the eversion differ between the two groups of insects. For example, in three families of moths, eversion and retraction of pheromone glands are controlled by paired anterior and posterior apophyses and associated muscles (Percy and George, 1979). In contrast, the pheromone gland in *H. parallela* lacks such apophyses, and the associated muscles work mainly to retract the gland back into the abdominal cavity. Therefore, eversion appears to be accomplished by abdominal pressure. All three sets of muscles relax, which allows hemolymph to be pushed into the posterior abdomen in order to evert the structure.

The distribution of the exocrine tissue in the eversible gland is rather simple; a layer of columnar cells lies immediately interior to the cuticular surface, which is exposed during calling. The high concentration of pheromone titer obtained from the pheromone gland (PO) confirmed that these columnar cells are the center of pheromone production. The pheromone is probably emitted through the soft cuticles (EN and EP in Figure 2D), but it does not seem to pervade other abdominal tissues (Figure 3). It was thought that the paired accessory glands connected ventrally to the vagina are putative pheromone glands in some scarabaeid beetles (Snodgrass, 1935; Zamotailov, 1988; Coca-Abia and Martin-Piera, 1991). In fact, these accessory glands in a melolonthine, *Costelytra zealandica*, harbor a symbiotic bacterium that produces phenol, a sex attractant in the same species (Hoyt et al., 1971; Stringer, 1988). However, in *H. parallela*, the near absence of the major pheromone component in the homologous paired accessory glands demonstrated that they were not involved in pheromone production. Further investigation is necessary with respect to the true functions of these accessory glands in this species.

The pheromone-producing columnar cells were surrounded by a layer of

relatively thick soft cuticle. This layer appeared thicker than that of lepidopterans (Percy-Cunningham and MacDonald, 1987), but it stretched and increased its surface area when the entire gland engorged (Figures 1C and 2C). Pheromone compounds produced by the columnar cells supposedly permeate through this soft cuticle, cross the thin epicuticle, and are emitted to the exterior.

The eversible pheromone gland (PO) in *H. parallela* is likely to be an extension of a genital chamber (Snodgrass, 1935; Coca-Abia and Martin-Piera, 1991). From the perspective of ontogeny, the genital chamber is a union of a genital pore and membranous invaginations of abdominal integument, both of ectodermal origin. Since the latter are modified intersegmental membranes, the pheromone gland (a layer of columnar cells, Figure 2D and E) is essentially a derived cuticular epithelium. It is known in *Anomala* spp. (Scarabaeidae: Rutelinae) that abdominal cuticular epithelium serves as a pheromone gland (Tada and Leal, 1997). Therefore, the superficial dissimilarities of pheromone glands and their localization between some anomalan and melolonthine beetles do not imply a large ontogenic gap between these taxa. In fact, *Heptophylla picea*, which belongs to Melolonthinae, has a pheromone gland of the anomalan-type, and the extract of a genital chamber pulled out from an abdominal tip did not contain pheromone (Tada and Leal, 1997). It will be interesting to investigate further the degrees of the morphological diversity of pheromone glands among different groups of Melolonthinae.

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EVIDENCE FOR ADDITIONAL PHEROMONAL COMPONENTS MEDIATING OVIPOSITION AGGREGATION IN *Schistocerca gregaria*

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Abstract—Oviposition responses of gregarious adult gravid females of the desert locust *Schistocerca gregaria* to sand previously used for oviposition by gregarious females (contaminated sand, with eggs and froth removed) and to sterilized sand (noncontaminated sand) were compared in bioassays. Gregarious females preferentially oviposited into contaminated sand. The number of egg pods laid by the females was found to correlate with the frequency of use of the contaminated sand for oviposition. Charcoal-trapped volatiles from the contaminated sand also elicited similar responses from gregarious females. Gas chromatography–electroantennographic detection (GC-EAD) analysis of the trapped volatiles revealed the presence of three electrophysiologically active compounds that were identified by GC-MS as (*Z*)-6-octen-2-one, (*E,E*)-3,5-octadien-2-one and its geometric isomer (*E,Z*)-3,5-octadien-2-one. The relative amounts of the three EAG-active compounds in the volatiles increased with consecutive oviposition by the gregarious females. These results indicate mediation of additional pheromonal components in the oviposition behavior of gregarious desert locust that are distinct from those that were previously identified in the froth volatiles.

Key Words—*Schistocerca gregaria*, oviposition, pheromone, locust, 6-octen-2-one, 3,5-octadien-2-one.

INTRODUCTION

Group oviposition in gregarious locusts is mediated by pheromones (Norris, 1963, 1970; Lauga and Hatte, 1977, 1978; Saini et al., 1995; Rai et al., 1997).

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In the migratory locust, *Locusta migratoria* L., this is mediated by a pheromone present in the sand into which gregarious females had previously laid (Lauga and Hatte, 1977, 1978). The pheromone level in sand increased with repeated layings by adult females, thus increasing its attractiveness. The activity of the sand was reported to last for as long as six months (Lauga and Hatte, 1977, 1978). However, the chemical identity of the pheromone is unknown. In the desert locust *Schistocerca gregaria* Forskål, gregarious females ready to lay are attracted to a common egg-laying site by acetophenone and veratrole emitted in the volatiles of froth of egg pods deposited by earlier laying conspecifics (Saini et al., 1995; Rai et al., 1997). Additional oviposition pheromones of low volatility from the froth, hypothesized to be contact signals, also were implicated in the oviposition behavior of this locust (Saini et al., 1995). Our detailed observations in the laboratory on the oviposition behavior of the gregarious desert locust suggested that females made their oviposition-site selection based on additional signals associated with the sand. Indeed, ovipositing females were seen touching the sand with their antennae, palpi, and mouth parts and preferentially ovipositing where conspecifics had previously laid, similar to observations made earlier in *L. migratoria* (Kennedy, 1949; Lauga and Hatte, 1977, 1978) and *S. gregaria* (Popov, 1958; Saini et al., 1995). The present study was undertaken to explore whether additional signals present in contaminated sand were involved in the oviposition behavior of the gregarious phase of this locust.

METHODS AND MATERIALS

Insects. Gregarious *S. gregaria* were reared under crowded conditions—200–300 individuals in aluminum cages measuring 50 × 50 × 50 cm (Obeng-Ofori et al., 1993)—and were provided with fresh wheat seedlings and bran as food.

Preparation of Contaminated Sand for Bioassays. Sand, washed thoroughly with water and air-dried, was heat-sterilized by baking in an oven at 170°C for 24 hr. After cooling to room temperature, the purity of the sand was checked for the presence of volatile contaminants by collection and analysis of its volatiles as described below. Sterilized sand was moistened with water to give a moisture content of 15% (15 ml of water added to 100 g of sand) (Norris, 1968). Eight aluminum oviposition cups (10 cm long × 4 cm ID) were filled with the sand and placed at the front of the false floor of standard aluminum locust rearing cages (50 × 50 × 50 cm) containing gravid females (Saini et al., 1995). After 24 hr, any deposited egg pods were removed and the contents of the cups transferred onto a metal tray covered with a sheet of aluminum foil to air dry overnight in a temperature-controlled room maintained at 30 ± 2°C. After drying, the sand was sifted through a wire mesh (2 mm^2) to remove remnants

of dried eggs and froth. The moisture content of the sifted sand was restored to its original level of 15% by adding an appropriate amount of water before subsequent use in assays as contaminated sand. In experiments where the test sand was exposed to multiple oviposition, egg pods were removed after each oviposition and the sand air-dried, sifted to remove remnants of the egg pods, and moistened as described above.

Behavioral Assays. Two choice bioassays were carried out in standard aluminum cages (20 × 20 × 20 cm) with a sliding glass front, wire gauze on the top, bottom, and two sides, and an aluminum sheet at the back. The four corners of the floor had round holes into which aluminum oviposition cups were fitted (Saini et al., 1995). Before assays, two diagonally facing holes were blocked by placing the oviposition cups in them upside down. The remaining two cups were filled with sand and used for the assays; one cup was the source of the test stimulus, and the other was a control.

Oviposition responses of gregarious gravid females (four in each replicate) were determined in the following situations: (1) a choice between noncontaminated sand (sterilized moistened sand) and contaminated sand obtained from the preparation described above (sand into which females had laid a maximum 10 egg pods); (2) a choice between noncontaminated sand and sand into which females had laid cumulatively in consecutive experiments, and into which 15, 20, 25, and 30 egg pods, respectively, had been laid; (3) a choice between noncontaminated sand and sand loaded with charcoal-trapped volatile extract from contaminated sand at two different doses, equivalent to three and six egg pods. The volatile extract was prepared from a dichloromethane stock solution, which was first added to 250 μl polyethylene glycol 200 (Aldrich Ltd.), for slow release, then to distilled water before mixing the mixture thoroughly with sterilized sand to give a moisture content of 15%. The control sand mixture (noncontaminated) was prepared similarly, with the same quantities of carriers but without the volatile extract. In all assays, cups containing both noncontaminated and contaminated sand were replaced daily and their positions alternated to avoid any positional bias. For bioassays with contaminated sand, each test was replicated 10 times. Tests with the volatile extract were replicated 36 times in the case of three egg pod equivalents and 18 times for six egg pod equivalents.

Oviposition response for each trial was monitored by counting the total number of egg pods laid in the control and treated cups, respectively. Differences in responses in each trial were tested by the chi-square test.

Collection of Volatiles. Volatiles were collected separately from four standard oviposition tubes each of sterilized sand (noncontaminated sand) and contaminated sand (all eggs and froth removed from the sand by sifting through 2-mm^2 wire mesh). The sand was placed in a glass chamber (10 cm long × 3.5 cm ID) and aerated (Assad et al., 1997). A charcoal-filtered airstream (106 ml/min) from a compressed air cylinder was passed over the sand for 24 hr in

a room maintained at 30 ± 2°C. The volatiles were collected on charcoal (60 mg) traps contained in Pyrex glass tubes (Torto et al., 1994). Collections were done from: (1) air-dried sterilized sand; (2) sand from (1) containing 15% moisture; (3) air-dried contaminated sand; and (4) sand from (3) containing 15% moisture. The volatiles were eluted from the charcoal traps with HPLC-grade dichloromethane (2 ml, Aldrich Ltd.), each set pooled together, and stored in the freezer at −15°C until used.

In addition, volatiles were collected separately from 120 froth plugs and 120 egg masses that were obtained from the same egg pods to trace the sources of EAG-active compounds. The froth plugs and egg masses were each crushed in sterilized sand, moistened with water to give a moisture content of 15%, and the volatiles of each set collected as described above.

Analysis of Volatiles. Volatiles were concentrated under a stream of nitrogen at 0°C to 100 μl. Eight microliters of the extracts were analyzed by gas chromatography–electroantennographic detection (GC-EAD) (Arn et al., 1975) on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with an FID detector and a Syntech EAD system using conditions previously described (Torto et al., 1994). The GC oven was fitted with an HP capillary column (Carbowax 20 M, 50 m × 0.2 mm ID × 0.2 μm) and temperature programmed as follows: 60°C (for 10 min), then 5°C/min to 180°C, held for 5 min, and raised at 10°C/min to 220°C. FID and EAD signals were monitored synchronously (Torto et al., 1996) using a program on a GC–EAD interface card (Syntech) installed in a PC (Harvard Professional Computer, American Megatrends, Inc.). The signals were recorded at a fixed speed of 9.2 cm/min and stored as pages of 128-sec duration. One complete run using the GC-EAD conditions generates 25 pages. In addition, the FID signal was plotted on a HP 3396 Series II integrator at 0.5 cm/min. GC-MS analyses were carried out on a VG Masslab 12-250 mass spectrometer (EI, 70 eV) coupled to a HP5790 gas chromatograph.

Compounds were identified by comparing their spectral data with those in the library (NIST Registry of Mass Spectral Data, 1995) of the mass spectrometer. The relative amounts of electrophysiologically active compounds were quantified in GC runs on a HP3396 Series II integrator using their peak areas.

RESULTS AND DISCUSSION

Our assays showed that gregarious females of *S. gregaria* oviposited more into sand previously laid in (contaminated) by conspecifics than into noncontaminated sand (Figure 1). Volatiles collected from contaminated sand also elicited similar responses from females (Figure 2).

Airborne volatiles from air-dried and moistened sterilized sand gave no significant detectable peaks. In the analyses of the trapped volatiles from air-dried

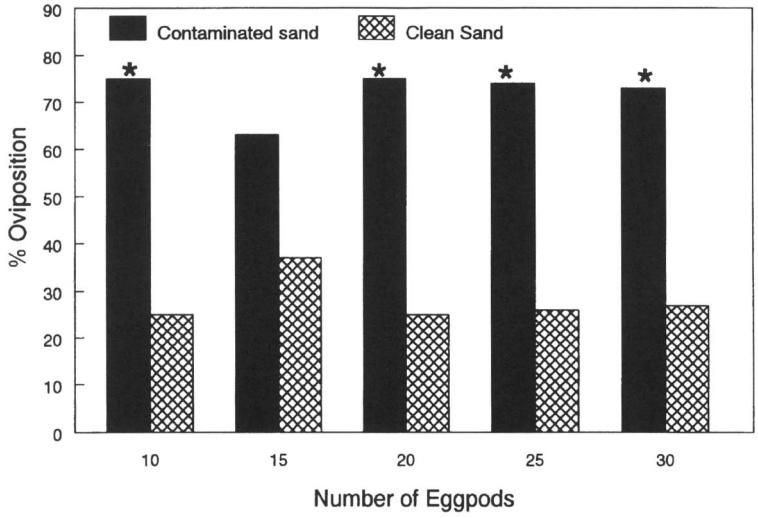

FIG. 1. Oviposition responses of gregarious females of *S. gregaria* to sand contaminated by conspecifics with different egg pod loadings in a choice bioassay. Solid bars represent responses to contaminated sand, while cross-hatched bars represent responses to noncontaminated sand control. Difference from control (chi-square test) indicated by $*P < 0.05$.

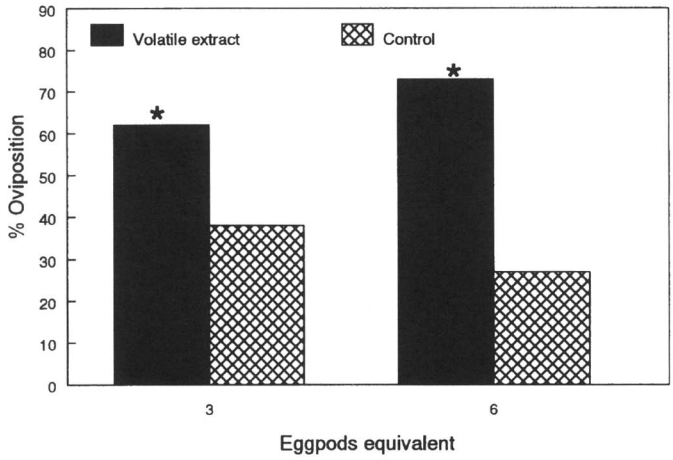

FIG. 2. Oviposition responses of gregarious females of *S. gregaria* to sterilized sand contaminated with the volatile extract of sand into which females had laid. Solid bars represent responses to contaminated sand; cross-hatched bars represent responses to noncontaminated sand control. Difference from control (chi-square test) indicated by $*P < 0.05$.

contaminated sand and moistened contaminated sand, the latter gave consistently more peaks than the former (Figure 3A and 3B). Three eluting compounds, represented by peaks **1–3**, were consistently electrophysiologically active in GC-EAD analysis of the volatiles of moistened contaminated sand (Figures 3 and 4). The relative amounts of the three compounds increased in the volatiles with increased use of the sand for oviposition (Figure 3, Table 1). GC-MS analysis allowed the characterization of all the three compounds as unsaturated aliphatic ketones (Figure 5), and they were identified by comparison of their mass spectral data to library data (NIST Registry of Mass Spectral Data, 1995), as follows: **1**, (*Z*)-6-octen-2-one, *m/z* (%) at 43 (100), 41 (35), 68 (29), 39 (22), 55 (21), 67 (16), 69 (10), 58 (8), 97 (8), 126 (3), 108 (3), 111 (2); **2**, (*E,E*)-3,5-octadien-2-one, *m/z* (%) at 95 (100), 43 (82), 81 (55), 39 (45), 79 (42), 53 (39), 41 (34), 124 (22), 51 (19), 55 (14), 65 (13), 109 (13), 77 (12), 50 (11), 66 (11), 52 (10); and **3**; (*E,Z*)-3,5-octadien-2-one, *m/z* (%) at 95 (100), 43 (61), 81 (46), 39 (37), 53 (33), 79 (31), 41 (27), 51 (16), 124 (14), 65 (11), 109 (10). Comparison with authentic samples is needed to confirm the structures of these compounds.

In previous studies on oviposition of the desert locust, the froth plug from the egg pod laid by gregarious females was shown to be a source of chemical signals inducing egg laying in conspecifics (Saini et al., 1995). In the present study, sand (without the egg pods) used for previous egg laying by gregarious females also induced egg laying in conspecifics, in agreement with results obtained with ovipositing females of the migratory locust *Locusta migratoria* (Lauga and Hatte, 1977, 1978). Surprisingly, the candidate compounds that we identified from the contaminated sand were different from the oviposition-inducing compounds, acetophenone and veratrole, previously identified from the froth (Saini et al., 1995; Rai et al., 1997). However, our results confirm previous hypotheses on the involvement of additional pheromone components in the oviposition behavior of this locust species (Saini et al., 1995).

Our results suggest selective adsorption of certain compounds including the EAG-active compounds (*Z*)-6-octen-2-one, (*E,E*)-3-5-octadien-2-one and its geometric isomer to sand granules compared to other egg pod volatile derivatives released at the time of oviposition. This is confirmed by the detection of higher levels of these compounds, particularly the three EAG-active compounds, in the volatiles of the sand after its repeated use for oviposition. A possible logical cause for selective adsorption of the compounds by the sand granules could be laboratory thermal-conditioning of the sand prior to the initial oviposition assay. This may have increased surface activation of the sand granules for strong adsorption of medium-polar and polar molecules. Whether these results can be extrapolated to the situation in the field remains to be determined. However, the relatively long periods of high day temperatures associated with the habitat of the desert locust may serve to modify the adsorption characteristics of sand granules for adsorption of volatile compounds and water. The observation that

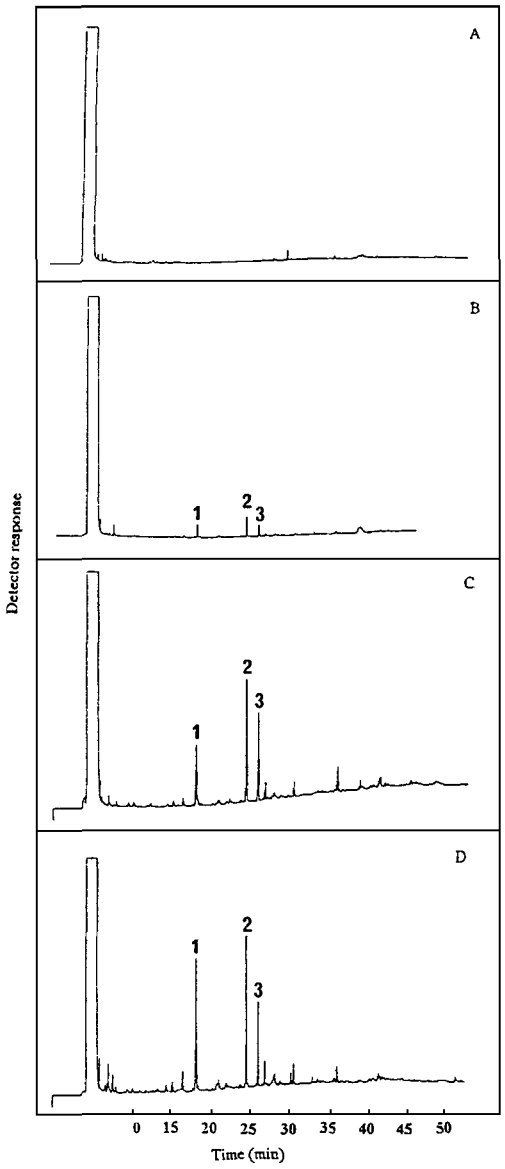

FIG. 3. Gas chromatograms of volatiles collected from (A) noncontaminated sand or air-dried contaminated sand (contaminated with 7 egg pods), and (B–D), moistened contaminated sand in which females had laid (B) 7, (C) 21, and (D) 35 egg pods. Electrophysiologically active peaks are labeled **1**, **2**, and **3**.

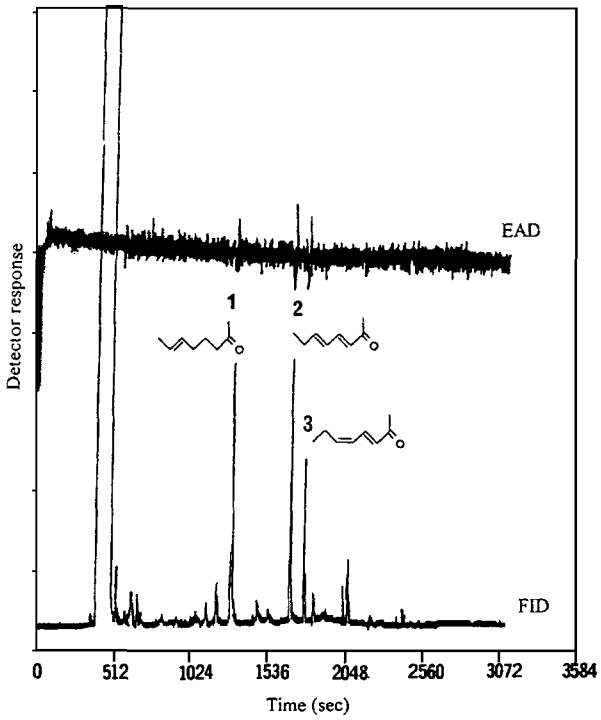

FIG. 4. Representative reconstructed computer GC-EAD recordings of female *S. gregaria* antennae, responding to compounds **1–3** in the crude volatile extract of sand contaminated by ovipositing gregarious females. **1**: (*Z*)-6-octen-2-one, **2**: (*E*,*E*)-3,5-octadien-2-one, and **3**: (*E*,*Z*)-3,5-octadien-2-one.

TABLE 1. GC PEAK AREAS OF EAD-ACTIVE COMPOUNDS IDENTIFIED IN VOLATILES OF SAND CONTAMINATED BY OVIPOSITING FEMALES OF GREGARIOUS *S. gregaria*

	GC peak area of compound		
Exp.[a]	(*Z*)-6-Octen-2-one	(*E*,*E*)-3,5-Octadien-2-one	(*E*,*Z*)-3,5-Octadien-2-one
I	11880 (1)[b]	15348 (1)	9190 (1)
III	15036 (1)	34678 (2)	26324 (3)
V	35009 (3)	55256 (4)	41083 (5)
VII	134932 (11)	121533 (8)	67619 (7)

[a]Oviposition experiments with sand contaminated with about 7 (I), 15 (III), 27 (V), and 35 egg pods (VII).
[b]Column values in parentheses are area ratios relative to that of first oviposition.

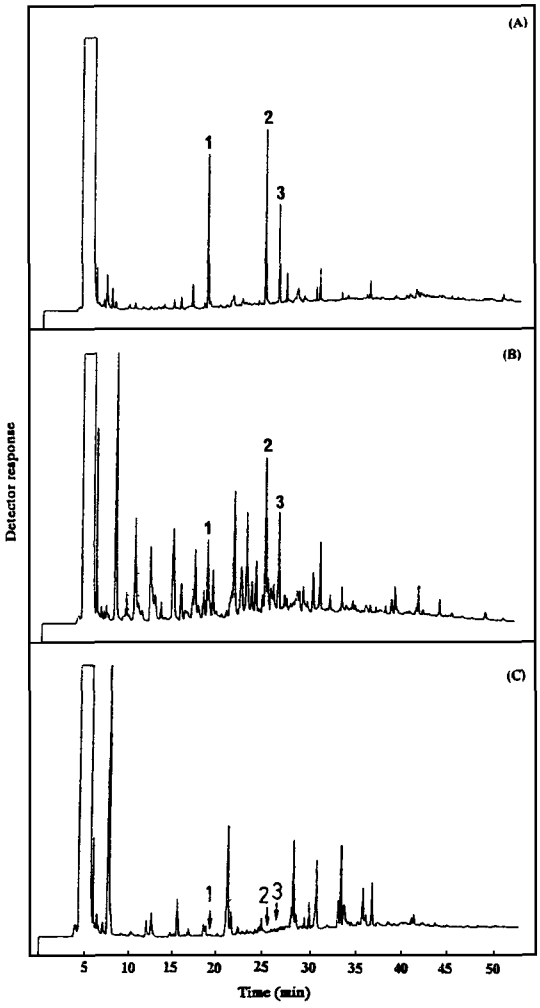

FIG. 5. Gas chromatograms of volatiles collected from (A) moistened contaminated sand, (B) eggs crushed in sterilized sand, and (C) froth plugs crushed in sterilized sand showing the levels of compounds **1**, **2**, and **3**.

gravid females antennate the sand prior to oviposition (Popov, 1958; Saini et al., 1995) suggests a search for specific compounds associated with the soil before laying.

Interestingly, entrainment of air-dried contaminated sand prior to moistening did not give detectable amounts of the ketones (Figure 3A; compare with

Figure 3B). Thus, the release of the compounds from sand appears to be associated with exchange with water molecules at the adsorbed sites. This would account for the fact that both the EAG-active and nonactive compounds are of medium polarity containing carbonyl groups.

Comparative analysis of volatiles derived from the eggs and the froth showed detectable amounts of the three ketones in the former, but only trace amounts in the latter (Figure 6). This suggests that these carbonyl compounds are not derived from the froth but are secreted into the sand directly from the eggs during oviposition. The underlying significance of this, if any, is unclear.

In conclusion, the present study has led to the identification of straight-chained unsaturated ketones as components of the oviposition pheromone system of gregarious-phase *S. gregaria*. The synthesis of the compounds is in progress. Their availability will facilitate a detailed study of the roles of different signals associated with group oviposition in *S. gregaria*, including the froth-derived benzene derivatives and the male-produced aggregation pheromone.

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CUCURBITACINS OF *Cucumis prophetarum* AND *Cucumis prophetarum*

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Abstract—Cucurbitacin B, isocucurbitacin B, dihydrocucurbitacin B, cucurbitacin E, dihydrocucurbitacin E, isocucurbitacin D, dihydroisocucurbitacin D, cucurbitacin I, dihydrocucurbitacin I, cucurbitacin Q1, and dihydrocucurbitacin Q1 were identified for the first time as constituents of *Cucumis prophetarum* L.. Cucurbitacin B, cucurbitacin O, cucurbitacin P, cucurbitacin Q1, dihydrocucurbitacin Q1, isocucurbitacin E, and dihydroisocucurbitacin E also were identified as constituents of *C. prophetarum* Jusl. ssp. Dissectus. Isocucurbitacin E and dihydroisocucurbitacin E were isolated for the first time in nature. The chemical structures were determined with extensive spectroscopic analysis including 2D NMR, ^{1}H NMR, ^{13}C NMR, correlated spectroscopy (COSY), heteronuclear chemical shift correlation (HETCOR), attached proton test (APT), and distortionless enhancement by polarization transfer (DEPT).

Key Words—Cucurbitacins, isocucurbitacin, dihydrocucurbitacin, *Cucumis prophetarum.*

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INTRODUCTION

The growing interest in cucurbitacins is the result of the wide range of activities they exhibit toward plants, insects, and animals. They are known for their extreme bitterness, mammalian toxicity, their purgative, emetic, and cytotoxic effects, and as a source of insect antifeedants (Lavie and Glotter, 1971, Halaweish, 1987; Miro, 1995). Recently, they were exploited for their antitumor properties, differential cytotoxicity toward renal, brain tumor, and melanoma cell lines (Fuller et al., 1994, Cardellina et al., 1990), inhibition of cell adhesion (Musza et al., 1989), and possible antifungal effects (Bar-Nun and Mayer, 1989, Tallamy et al., 1997).

Cucurbitacins are known as phagostimulants for diabroticite beetles, which can detect these compounds at quantities as low as 0.1 ng. This phenomenon has recently been exploited in toxic semiochemical baits (cucurbitacins laced with insecticide) to monitor and control several pest species of diabroticites (Metcalf et al., 1987; Lance, 1988; Weissling et al., 1989; Lance and Sutter, 1990). Since a number of diabroticites are serious pests of corn, cucurbits, peanuts, and beans, the impact of these beetles on agriculture worldwide is enormous. In the early 1980s, species such as the southern, western, and northern corn rootworms caused well over one billion dollars per year in yield loss and insecticide costs in the United States alone (Metcalf, 1994). The use of cucurbitacins as constituents of semiochemical baits for diabroticite control or as antifeedents against and/or deterrents to oviposition for nonadapted phytophagus insects has been hindered by the lack of economically attractive cucurbitacin sources.

In view of the importance of these compounds to medicine and agriculture, there is a need to elucidate the chemistry of cucurbitacin-bearing plants. In the course of our chemical investigation of cucurbitaceous plants, we have studied two closely related species: *Cucumis prophetarum* L. and *C. prophetarum* Jusl. AP. L. ssp. Dissectus (Naud.) Jeffery, which grow wild in the southeast part of Egypt. The two subspecies have some morphological differences between the flower parts and fruit shape. We wanted to investigate their chemical constituents for further applied studies.

METHODS AND MATERIALS

Melting point (mp) was determined on Fisher digital apparatus (model 355). Infrared (IR) spectra were recorded on a Perkin-Elmer 281P spectrophotometer, and ultraviolet (UV) spectra were measured with the Perkin-Elmer Lambda 3B spectrophotometer. NMR spectra were recorded on a Varian VXR 300A spectrometer (300 MHz for ^1H and 75 MHz for ^{13}C) in $CDCl_3$ for compounds **1**, **2**, **3**, and **7** and in CD_3OD for **4**, **5**, **6**, and **8**. A Waters 600 high-performance liquid

chromatography (HPLC) equipped with a Waters 486 UV detector and RP C_{18} column (7.8 mm, 25 cm) was used for purification of compounds. LC-MS was performed with a Waters 600 pump and Vestec Thermospray MS; ammonia was used as the ionizing gas.

Plant Material

Cucumis prophetarum L. (CPL) was collected from wild plants growing in the southeast part of Egypt near the Red Sea (Elaolbea Mountains) in March 1987. The plants were separated into fresh fruits, aerial parts, and roots, which were air-dried, powdered, and kept at 0°C.

Cucumis prophetarum Jusl. AP. L. Dissectus (Naud.) Jeffery (CPJ) fresh fruits were collected from the wild plants growing in the southeast corner of Egypt near the Red Sea (Mersa Halaieb) in January 1989. The two plants were identified by Dr. Nabil El-Hadidy, Department of Botany, Faculty of Science, Cairo University, Cairo, Egypt. A voucher specimen of each plant has been deposited in the Department of Pharmacognosy, Faculty of Pharmacy, University of Mansoura, Mansoura, Egypt.

Extraction

The fresh fruits of both plants (600 g each) were separately homogenized with 95% EtOH (2 liters × 4) and the EtOH was recovered. The total yields of the alcoholic extractives were 9.8% and 9.2% for CPL and CPJ, respectively. The air-dried powdered stems and leaves (1.5 kg) of CPL were extracted with 95% EtOH in a Soxhlet apparatus. The ethanol was removed by distillation to give a syrupy residue (262.5 g).

Fractionation and Separation

Fractionation of Alcoholic Extract of Fresh Fruits of CPL. The syrupy extract (58.8 g) was shaken with ether (250 ml × 4). During the concentration of the ether extract, a white precipitate was obtained. The precipitate was crystallized from an ether–MeOH mixture (99 : 1) to give 100 mg of white crystals, which were repurified with HPLC with system A [MeCN–H_2O (1 : 1)] and detected at 235 nm to afford cucurbitacin B (**1**), 80 mg; mp 184–186°C; IR (film) (cm^{-1}): 3400 (OH), 1740, 1240 (AC), 1690 (CO in six-membered ring), 1670 (α-β unsaturated ketone), and 1635 (*trans* olefinic absorption conjugated with CO); LC-MS: 576 [M^++18].

The ether extract (10 g) was chromatographed on a Silica gel column (360 g, 100–200 mesh) and fractions were eluted with petroleum ether, $CHCl_3$, and MeOH mixtures (gradient). We collected 230 fractions (50 ml each) and screened them by TLC with a solvent system of toluene–ethylacetate (3 : 1). Fractions

36–38 (eluted with petroleum ether–$CHCl_3$, 1 : 1) were pooled (0.3 g) and purified with HPLC using system A to afford compounds **2** (12 mg, R_t = 9.48) and **3** (25 mg, R_t = 11.48). Fractions 95–100 (eluted with $CHCl_3$–MeOH, 19 : 1) were combined (0.5 g) and purified by HPLC and system A to afford compounds **4** (40 mg, R_t = 14.11); **5** (15 mg, R_t = 17.12), and an unknown (3 mg, R_t = 22.32). Fractions 131–170 (eluted with $CHCl_3$–MeOH, 4 : 1) were combined (1.2 g) and the residue was crystallized from $CHCl_3$–MeOH mixture to give **9** (150 mg).

Isocucurbitacin B (2) and Dihydroisocucurbitacin B (2a). White crystals (MeOH); mp 226–230°C; IR (cm^{-1}): 3400, 1740, 1240, 1690, 1670; LC-MS: m/z 576 [M^++18] and m/z 578 [M^++18].

Cucurbitacin E (3) and dihydrocucurbitacin E (a). White crystals (MeOH); mp 230–230°C; IR (cm^{-1}): 3400, 1740, 1240, 1690, 1680, 1670, 1635; LC-MS: m/z 574 [M^++18] and 576 [M^++18].

Isocucurbitacin D(4) and Dihydroisocucurbitacin D (4a). White needle-shaped crystals; IR (cm^{-1}): 3400, 1690, 1670, 1635; LC-MS: m/z 534 [M^++18] and m/z 536 [M^++18].

Cucurbitacin I (5) and Dihydrocucurbitacin I (5a). White crystals; IR (cm^{-1}): 3400, 1690, 1680, 1670, 1635; LC-MS: m/z 532 [M^++18] and m/z 534 [M^++18].

Sitosterol-3-glucoside (9). White needle crystals (MeOH); mp 271°C; IR (cm^{-1}): 3400, 2950, 1640, 1450; MS: m/z 576 (11.7) [$C_{35}H_{60}O_6$], 414 (47), 397 (76), 273 (19), 271 (24.4), 255 (89), 229 (28.2), 213 (26), 175 (11.3), 161 (36.2), 109 (37.5), 107 (61.4), 83 (100). Acid hydrolysis of **9** (22 mg) with 5% HCl afforded glucose and sitosterol (17 mg, mp 140–142°C).

Fractionation of Alcoholic Extract of Leaves and Stems of CPL. The syrupy extract was partitioned with ether, and the ether extract (3 g) was chromatographed on a silica gel column (150 g) with toluene and EtOAc mixtures (gradient). We collected 608 fractions (10 ml each). Fractions 560–572 were eluted with toluene–ethylacetate (55 : 45), combined (0.1 g), and purified by HPLC with system A to afford compound **6** (15 mg, R 8.82) consisting of cucurbitacin Q1 (**6**) and dihydrocucurbitacin Q1 (**6a**). White crystals (MeOH); mp 234–236°C; IR (cm^{-1}): 3400, 1740, 1240, 1690, 1635; LC-MS: m/z 578 [M^++18] and m/z 580 [M^++18].

Fractionation of Alcoholic Extract of Fresh Fruits of CPJ. The syrupy extract (55.2 g) was partitioned with ether (250 ml × 5). During the process of concentration, a white precipitate (250 mg) was obtained and purified by HPLC with the solvent MeCN–H_2O (55 : 45) to afford five compounds: **1** (55 mg, R_t = 11.6), **7** (8 mg, R_t = 12.7), **6** (130 mg, R_t = 8.6), and **8** (7.5 mg, R_t = 6.9).

Isocucurbitacin E (7) and Dihydroisocucurbitacin E (7a). White needle crystals (MeOH); mp 192–194°C; IR (cm^{-1}): 3400, 1740, 1240, 1690, 1670, 1635, 1680; LC-MS: m/z 576 [M^++18] and m/z 574 [M^++18].

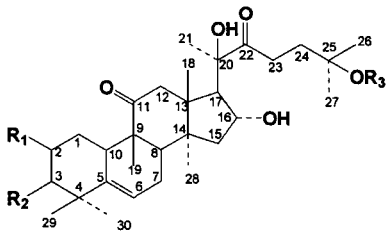
Cucurbitacin O (8) and Cucurbitacin P (8a). White needle crystals; mp

205°C; IR (cm^{-1}): 3400, 1690, 1670, 1635; LC-MS: m/z 536 [M^++18] and 538 [M^++18].

RESULTS AND DISCUSSION

Results of the TLC investigation as well as the ^1H and ^{13}C NMR spectra for the isolated compounds (**1–8**) and structures are summarized in Figure 1 and Tables 1–3.

Since the beginning of this investigation, it was clear that these two species contained a large number of components and that it would be difficult to separate them because of structural similarities between derivatives of the same skeleton. High-resolution NMR experiments and LC-MS were very helpful for the identification of the components, as was published data. Technical difficulties have



Cucurbitacin	No	R_1	R_2	R_3	$\Delta^{1,2}$	$\Delta^{23,24}$	$\Delta^{1,10}$
B	1	OH	C=O	Acetyl	-	+	-
Iso-B	2	C=O	OH	Acetyl	-	+	-
Dihydro-iso-	2a	C=O	OH	Acetyl	-	-	-
Cu-E	3	OH	C=O	Acetyl	+	+	-
Hihydro-E	3a	OH	C=O	Acetyl	+	-	-
Iso-D	4	C=O	OH	-	-	+	-
Dihydro-iso-	4a	C=O	OH	-	-	-	-
Cu-I	5	OH	C=O	-	+	+	-
Dihydro-I	5a	OH	C=O	-	+	-	-
Q1	6	OH	OH	Acetyl	-	+	-
Dihydro-Q1	6a	OH	OH	Acetyl	-	-	-
Iso-E	7	C=O	OH	Acetyl	-	+	+
Dihydro-iso-	7a	C=O	OH	Acetyl	-	-	+
O	8	OH	OH	-	-	+	-
P	8a	OH	OH	-	-	-	-

FIG. 1. Cucurbitacins isolated from *Cucumis prophetarum* L. and *C. prophetarum* Jusl. AP.L.ssp. Dissectus (Naud.) Jeffery.

TABLE 1. RESULTS OF TLC[a] INVESTIGATION OF COMPOUNDS ISOLATED FROM *Cucumis prophetarum* L. AND *C. prophetarum* JUSL. AP.L. SSP. DISSECTUS (NAUD.) JEFFERY

Compound	R_f[b]	Spray reagent[c]			
		A	B	C	D
1	0.23	Reddish brown	Brick red	Orange	
2	0.44	Reddish brown	Brick red	Yellow-orange	
2a	0.45	Brownish yellow	EGF[d]	Yellow-orange	
3	0.39	Reddish brown	Brick red	Yellow-orange	Brown
3a	0.40	Brownish yellow	EGF	Yellow-orange	Brown
4	0.15	Reddish brown	Brick red	Orange	
4a	0.14	Brownish yellow	EGF	Orange	
5	0.13	Reddish brown	Brick red	Orange	Brown
5a	0.12	Brownish yellow	EGF	Orange	Brown
6	0.06	Reddish pink	Brick red		
6a	0.07	Brownish yellow	EGF		
7	0.30	Brownish yellow	EGF	Yellow-orange	Brown
7a	0.29	Reddish brown	Brick red		
8	0.01	Reddish pink	Brick red		
8a	0.02	Brownish yellow	EGF		

[a]Silica Gel G plates; solvent system: toluene–ethyl acetate (6 : 4).
[b]R_f = retention factor.
[c]A = vanillin/phosphoric acid reagent (visible light); B = vanillin/phosphoric acid reagent (UV long range); C = triphenyl tetrazolium chloride reagent; D = ferric chloride reagent.
[d]EGF = emerald green fluorescence.

been reported (Jacobs et al., 1990) for the identification of similar compounds such as their dihydroderivatives from *Cayaponia* species because of the difficulty of resolving the normal cucurbitacins from either their dihydro and/or iso derivatives.

Compound **1** was isolated from both plants and identified as cucurbitacin B from spectral analysis (IR, LC-MS, and ^1H and ^{13}C NMR). Spectral data were in agreement with published results for cucurbitacins (Che et al., 1985; Velde and Lavie, 1983). Cucurbitacin B was taken as a reference guide in spectral analysis since it is one of the most widely distributed cucurbitacins.

Visualization of TLC plates with vanillin/phosphoric acid reagent (Table 1) showed that cucurbitacins give a brick-red color under long-range UV and reddish brown in visible light. On the other hand, dihydrocucurbitacins give emerald green fluorescence in long-range UV and brownish yellow in visible light. This characteristic was very helpful to distinguish cucurbitacins from their corresponding dihydroderivatives.

The LC-MS of compound **2** showed two molecular ion peaks at m/z 576 [M^++18] and m/z 578 [M^++18], which agree with the molecular formulas $C_{32}H_{46}O_8$ and $C_{32}H_{48}O_8$, respectively. A sharp singlet at δ 3.9 was assigned

TABLE 2. ^{1}H NMR DATA FOR ISOLATED CUCURBITACINS FROM *Cucumis prophetarum* L. AND *C. prophetarum* JUSL. AP.L.SSP. DISSECTUS (NAUD.) JEFFERY[a]

Cucurbitacin	H1α	H1b	H-2	H-3	H-6	H-10	H-23	H-24
Cu B (**1**)	2.15–2.42 m	1.01 m	4.28 m		5.73 m	2.69 brd (13.6)	6.43 d (15.5)	7.02 d (15.5)
Iso-B (**2**)	2.22 dd (13.12)	1.03 m		3.89 s	6.93 m	2.75 m	6.43 d (15.5)	7.04 d (15.5)
Dihydroiso-B (**2a**)	2.22 dd (13.12)	1.03		3.89 s	5.93 m	2.75 brd	2.40 m 2.81 m	1.98 m 3.34 m
Cu E (**3**)	5.94 d (2.5)				5.76 m	3.49 brd	6.45 d (15.5)	7.05 d (15.5)
Dihydro-E (**3a**)	5.94 d (2.5)				5.76 m	3.49 brd	2.42 m 2.82 m	1.97 m 3.5 m
Iso D (**4**)	2.27 dd (13.12)	1.37 m		3.95 s	5.97 m	2.81 brd	6.8 d (15.5)	6.69 d (15.5)
Dihydroiso-D (**4a**)	2.27 dd (13.12)	1.37 m		3.95 s	5.97 m	2.81 m	2.54 m 2.74 m	1.72 m 3.31 m
Cu I (**5**)	5.75 d (2.5)				5.84 m	2.55 brd (13.5)	6.83 d (15.5)	6.98 d (15.5)
Dihydro-I (**5a**)	5.75 d (2.5)				5.84 m	3.34 d (14.5)	2.42 m 2.83 m	1.97 m 3.32 m
Q1 (**6**)	2.35–2.43 m	1.04 m	4.43 m	3.51 m	5.76 m	3.49 brd	6.83 d (15.5)	6.98 d (15.5)
Digydro-Q1 (**6a**)	2.35–2.43 m	1.04 m	4.43 m	3.51 m	5.76 m	3.49 brd	2.44 m 2.81 m	1.94 m 3.53 m
Iso E (**7**)	6.28 s			3.48 s	5.78 m		6.46 d (15.5)	7.06 d (15.5)
Dihydro-Iso E (**7a**)	6.28 s			3.48 s	5.78 m		2.43 m 2.82 m	1.81 m 3.32 m
Cu O (**8**)	2.34–2.4 m	1.04 m	4.42 m	3.50 m	5.74 m	3.5 brd (14)	6.82 d (15.5)	6.97 d (15.5)
Cu P (**8a**)	2.34–2.4 m	1.04 m	4.42 m	3.50 m	5.74 m	3.5 brd (14)	2.42 m 2.83 m	1.97 m 3.22 m

[a]d = doublet; dd = double doublet, m = multiplet; s = singlet and t = triplet.
Values in parentheses are coupling constants (J).

TABLE 3. ^{13}C NMR DATA FOR ISOLATED CUCURBITACINS FROM *Cucumis prophetarum* L. AND *C. prophetarum* JUSL. AP.L.SSP. DISSECTUS (NAUD.) JEFFERY

Compound	C-1	C-2	C-3	C-5	C-6	C-10	C-23	C-24
1	37.96 d[a]	71.61 d	213.02 s	140.34 s	120.41 d	33.68 d	120.32	151.84 d
2	38.79 d	210.59 d	80.20 d	138.16 s	120.27 d	36.27 d	121.88 d	152.07 d
2a	38.79 d	210.58 d	80.19 d	138.16 s	120.27 d	36.27 d	30.63 t	34.78 t
3	114.82 d	144.56 s	198.67 s	136.75 s	120.78 d	34.69 d	120.36 d	151.93 d
3a	114.82 d	144.56	198.67	140.40 s	120.73 d	34.87 d	30.66 t	34.77 s
4	40.26 t	215.26 s	81.43 d	139.97 d	121.28 d	37.39 d	122.67 d	155.35 d
4a	40.26 t	215.26 s	80.78 d	139.97 s	121.28 d	37.39 d	33.11 t	38.12 t
5	116.68 d	146.81 s	199.99 s	140.01 s	121.39 d	36.04 d	121.77 d	155.40 d
5a	116.78 d	146.80 s	199.99 s	140.01 s	21.38 d	37.43 d	33.15 t	38.13 t
6	34.76 t	71.98 d	81.89 d	142.70 s	119.94 d	34.92 d	122.67 d	151.58 d
6a	34.76 t	71.59 d	81.89 d	142.70 s	119.94 d	34.92 d	32.84 t	35.81 t
7	35.99 t	213.88 s	71.63 d	140.40 s	120.42 d	140.46 s	120.32 d	151.54 d
7a	35.99 t	213.88 s	71.63 d	140.40 s	120.42 d	140.46 s	30.67 t	34.82 t
8	34.79 t	71.62 d	81.93 d	142.71 s	119.97 d	34.96 d	122.62 d	152.58 d
8a	34.78 t	71.62 d	81.93 d	142.71 s	119.97 d	34.96 d	33.12 t	38.14 t

[a]d = doublet; dd = double doublet, m = multiplet; s = single and t = triplet.

for H-3 and is known for isocucurbitacins (Jacobs et al., 1990; Halaweish, 1993; Hylands and Maged, 1986). The attached proton test (APT) and the distortionless enhancement by polarization transfer (DEPT) experiments indicated a mixture of 3 : 2 ratio and showed 10 methylenes (four signals, each corresponding to one methylene, and three signals, each corresponding to two methylenes); 12 methines (six signals, each corresponding to one methine, and three signals, each corresponding to two methines); 18 methyl groups (six signals, each corresponding to one methyl group, and six signals, each corresponding to two methyl). The extra low field signals at δ 30.64 and 34.78 were assigned to C-23 and C-24 of a dihydrocucurbitacin. The low intensity signals at δ 213.61 and 81.22 were attributed to C-22 and C-25, and showed a difference of 10.5 and 2 ppm due to the hydrogenation at C-23, 24, as they normally appear in cucurbitacin B (Che et al., 1985). The rest of the data were in agreement with the reference cucurbitacin B. Therefore, compound **2** was identified as a mixture of isocucurbitacin B (**2**) and dihydroisocucurbitacin B (**2a**).

Compound **3** was characterized as a mixture of cucurbitacin E and dihydrocucurbitacin E based on color reactions (Table 1), NMR data (Tables 2 and 3) and LC-MS. The latter showed a mixture of two compounds (3 : 2 ratio): m/z 574 [M^++18] and m/z 576 [M^++18] corresponding to formulas $C_{32}H_{44}O_8$ and $C_{32}H_{46}O_8$, respectively. A normal H-1,2 shift at δ 5.94 (vinyl proton, as doublet, J = 2.5 Hz) characterized the diosphenol nature in ring A assigned for $\Delta^{1,2}$ found in cucurbitacin E (Che et al., 1985). An acetate signal was clear from H and C shifts at 2.1 and 170 ppm, respectively. A dihydroderivative of cucurbitacin in the mixture was assigned from signals at δ 30.77 and 34.77, accommodated for C-23 and C-24, respectively. The effect of hydrogenation on $\Delta^{23,24}$ was clear from the downfield shift of C-22 and C-25 at δ 214.07 and at 81.25 by 11.5 and 2 ppm, respectively. High-resolution NMR experiments, APT, and DEPT data showed this compound to be a mixture in a 3 : 2 ratio. Dihydrocucurbitacin was assigned from ^{1}H and ^{13}C shifts (Tables 1 and 2). Proton and carbon connectivities were also confirmed from heteronuclear chemical shift correlation (HETCOR) and COSY experiments. These data together with comparison of published data (Jacobs et al., 1990; Che et al., 1985; Stuppner et al., 1991) allowed us to characterize this compound as a mixture of cucurbitacin E (**3**) and dihydrocucurbitacin E (**3a**).

Compound **4** was isolated as a mixture of an isocucurbitacin and its dihydroderivative as indicated from reaction with vanillin/phosphoric acid reagent and LC-MS that showed two molecular ion peaks at m/z 534 [M^++18] and m/z 536 [M^++18], corresponding to molecular formulas of $C_{30}H_{44}O_7$ and $C_{30}H_{46}O_7$, respectively. NMR experiments (Tables 2 and 3) showed an isocucurbitacin H-3 sharp singlet at δ 3.95 (Hylands and Maged, 1986; Halaweish, 1993; Velde and Lavie, 1983) as well as the disappearance of the H-2 signal at δ 4.39 in normal cucurbitacins. APT and DEPT revealed 10 methylenes (four signals, each

corresponding to one methylene each and three signals, each corresponding to two methylenes), 14 methines (six signals, each corresponding to one methine and four signals, each corresponding to two methines), and 16 methyls (four signals, each corresponding to one methyl and six signals, each corresponding to two methyls). Extra low intensity signals at 31.11 and 38.12 ppm in ^{13}C NMR were assigned to C-23 and C-24, respectively. The saturated bone in the side chain also shifted the C-22 (δ 217.16) carbonyl down field by 12 ppm, as it should normally appear in cucurbitacin D (Stuppner et al., 1991; Jacobs et al., 1990). The mixture was confirmed by COSY and HETCOR experiments. These data defined compound **4** as isocucurbitacin D (**4**) and dihydroisocucurbitacin D (isocucurbitacin R) (**4a**).

Compound **5** was a mixture of two cucurbitacins (3 : 2 ratio) as indicated from LC-MS, which showed two molecular ion peaks at m/z 532 [M^++18] and 534 [M^++18] corresponding to molecular formulas $C_{30}H_{42}O_7$ and $C_{30}H_{44}O_7$, respectively. The dihydroderivatives and diosphenol character of this compound were suggested from the color reaction with vanillin/phosphoric acid reagent. The proton spectrum showed a vinyl signal at δ 5.94 (d, J = 2.5 Hz) attributed to H-1 in $\Delta^{1,2}$ and indicated a diosphenol system in ring A. ^{13}C NMR showed low intensity signals at δ 33.15 and 38.13, assigned for C-23 and C-24, respectively, in a saturated $\Delta^{23,24}$. A downfield shift at δ 216.19 of C-22 carbonyl by 12 ppm is also due to bond saturation. This combined physical, chemical, and spectral evidence, together with comparison of published data allowed us to identify compound **5** as cucurbitacin I (**5**) and dihydrocucurbitacin I (**5a**) (Che et al., 1985; Velde and Lavie, 1983; Stuppner et al., 1991; Jacobs et al., 1990).

Compound **6** was a mixture of two compounds (3 : 2 ratio) as indicated from LC-MS and the color reaction with vanillin/phosphoric reagent. LC-MS showed two molecular ion peaks at m/z 578 [M^++18] and 580 [M^++18] corresponding to molecular formulas $C_{32}H_{48}O_8$ and $C_{32}H_{50}O_8$, respectively, and suggested a cucurbitacin and its dihydroderivative. The absence of carbonyl in ring A was clear with the existence of an extra oxygenated carbon as indicated from the NMR spectra (APT and DEPT). H-3 was observed at δ 3.51 as a doublet, which is due to the presence of another proton at C-2. This suggested a diol system in ring A, which is known for some cucurbitacins. The extra low intensity carbon signals at δ 32.84 and 35.81 in the ^{13}C spectrum were assigned to a saturated C-23 and C-24, respectively. Downfield shift of C-22 carbonyl and C-25 at δ 216.54 and 83.84 by 11.5 and 2 ppm are due to saturation of $\Delta^{23,24}$. Further structure identification was achieved by comparison with published data for cucurbitacin Q1 (Stuppner et al., 1991; Atta-Ur-Rahman et al., 1973). Therefore, compound **6** was identified as cucurbitacin Q1 (**6**) and dihydrocucurbitacin Q1 (**6a**).

LC-MS of compound **7** showed two molecular ion peaks at m/z 574 [M^++18] and 576 [M^++18] corresponding to molecular formulas $C_{32}H_{44}O_8$ and $C_{32}H_{46}O_8$, respectively, and suggesting a cucurbitacin with its dihydroderiva-

tive in a ratio of 1 : 3. This compound showed an isocucurbitacin character and two mass units more than cucurbitacin E. Reaction with visualizing agents suggested this compound was a diosphenol derivative. The ^{1}H NMR spectra showed a sharp singlet at δ 3.49, assigned to a single proton on an oxygenated carbon and accommodated for H-3. Therefore, the only position to accommodate an iso and diosphenol system tentatively in ring A is $\Delta^{1,10}$ and the C-2 carbonyl. The latter assumption was supported by the existence of only one vinyl proton as a singlet at δ 6.29 (H-1 normally shift at δ 5.9 as doublet). The carbon shifts of two quaternary carbons (APT) in an olefinic center at δ 140.46 and 140.4 were assigned to C-5 and C-10, respectively. The shift of C-2 carbonyl appearing upfield at δ 213.88 suggested α–β unsaturation. Based on these physical, chemical, and spectral data compound **7** could be assigned tentatively as dihydroisocucurbitacin E (**7**) and isocucurbitacin E (**7a**).

Compound **8** was a mixture of cucurbitacin O and its dihydroderivative (cucurbitacin P) based upon color reaction and LC-MS. The latter showed two molecular ions at m/z 536 [M^++18] and 538 [M^++18], corresponding to molecular formulas $C_{30}H_{46}O_7$ and $C_{30}H_{48}O_7$, respectively. The dihydro carbon signals at δ 33.1 and 38.14 were assigned to the C-23 and C-24 methylenes, respectively. In addition, the C-22 carbonyl (215.17 ppm) showed a down field shift of 10 ppm due to the saturation of $\Delta k^{23,24}$. These data compare favorably with the published data for cucurbitacin O and P (Che et al., 1985; Jacobs et al., 1990; Stuppner et al., 1991). Therefore, compound **8** was assigned as cucurbitacin O (**8**) and P(**8a**).

Compound **9** gave a rose-red color with the vanillin/phosphoric acid reagent and a positive Liebermann-Burchard's test, indicating its steroidal or triterpenoidal nature. The MS spectrum showed m/z 576 in accordance with formula $C_{35}H_{60}O_6$ and fragments at m/z 273, 271, and 255, indicating the presence of the steroidal nucleus with a single double bond (Zoretskii, 1976). The MS fragment at m/z 414 is due to a loss of 162 mass units of a sugar moiety (180–18) while fragments at m/z 273 and 255 are due to successive split of the side chain and water, respectively. Acid hydrolysis of compound **9** gave glucose and sitosterol. The aglycone was identified by comparison with authentic sitosterol (cochromatography and mp) and by comparison of its spectral data (Akihisha et al., 1987; Nes et al., 1992). Comparison of the glycoside physical and spectral data matched published results for sitosterol-3-*O*-glucoside (Garg and Nes, 1986; Akihisha et al., 1987; Nes et al., 1992).

In conclusion, this study represents the first report on the isolation and identification of cucurbitacin E; dihydrocucurbitacins E, I, and Q1; isocucurbitacins B and D; and dihydrocucurbitacins and -D (cucurbitacin R) from *Cucumis prophetarum* L. Dihydrocucurbitacins Q1 and E were identified for the first time in nature. Cucurbitacins B, Q1, O, and P; isocucurbitacin-E, and dihydrocucurbitacin-E were reported for the first time from *Cucumis prophetarum* Just Ap. L. ssp. Dissectus

(Naud) Jeffrey. Also, isocucurbitacin E and dihydro-iso-E were identified for the first time in nature.

The presence of isocucurbitacins with normal cucurbitacins supported the natural occurrence of these compounds, and they are not artifacts as once thought. The existence of the dihydrocompounds with their unsaturated derivatives also indicated the close relation and complexity of this series.

Cucurbitacins containing bait have been produced commercially (MicroFlo Company) and used successfully as a semiochemical bait to control cucumber beetles. The main hurdle for large-scale application is the presence of a suitable source for cucurbitacins, because of variation in quality and quantity of cucurbitacins in plants.

The existence of this diversity of cucurbitacins provides new material for optimizing cucurbitacin bait efficiency for control of diabroticites and for further development in antitumor applications.

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ALTERED CONSTITUTIVE AND INDUCIBLE PHLOEM MONOTERPENES FOLLOWING NATURAL DEFOLIATION OF JACK PINE: IMPLICATIONS TO HOST MEDIATED INTERGUILD INTERACTIONS AND PLANT DEFENSE THEORIES

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Abstract—Foliar feeding by the jack pine budworm *Choristoneura pinus pinus* altered the constitutive and induced monoterpene content and composition of *Pinus banksiana* phloem. In constitutive phloem tissue the ratios of α-pinene to β-pinene decreased with defoliation intensity. Biochemical responses to simulated bark beetle attack were also influenced by defoliation intensity. Total monoterpene concentrations were always higher in induced than constitutive tissue, but the extent and rate of monoterpene accumulation, and also proportionate changes in monoterpenes during induction, varied with defoliation intensity. Quantitative and compositional changes in response to simulated bark beetle attack were also affected by host seasonal phenology and time since defoliation. There was a positive relationship between myrcene concentration and arrival by the bark beetle *Ips grandicollis*. Bark beetle arrival rates were more strongly associated with the chemical composition of constitutive than reaction phloem chemistry. However, chemical changes during induced host responses were also associated with beetle behavior. The ratio of α-pinene to β-pinene during the first three days of induction was consistently associated with both defoliation by *C. pinus pinus* and susceptibility to *I. grandicollis*. These results show that direct injury to one plant tissue, foliage, cannot only cause chemical changes in another tissue, phloem, but such changes can be greater in the tissue not directly affected. The influence of defoliation intensity on monoterpene accumulation suggests a link between production and allocation of carbon within *P. banksiana*. The relationship between defoliation level and induced monoterpene concentrations was either parabolic

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or inverse, depending on the interval following defoliation. Likewise, the relationship between plant growth and defoliation intensity varied with the length of time following defoliation. Thus, plant defense theories can be improved by incorporating the length of time during which the effects of a stress are exerted.

Key Words—Induction, monoterpenes, defoliation, jack pine, jack pine budworm, bark beetles, interguild interactions, plant defense theories.

INTRODUCTION

A number of studies have demonstrated that feeding by insect herbivores can alter subsequent host suitability to other insect species (Rhoades, 1985; Hunter and Price, 1992; Power, 1992; Hunter and Schultz, 1993; Karban and Niibo, 1995). The direction of these changes can either benefit or detract from the second herbivore's success, depending on the plant and insect species involved, the extent and type of initial feeding, and features of the abiotic environment (Ericsson et al., 1980; Wood, 1982; Strong, 1983; Lewinsohn et al., 1991; Bause and Carisey, 1996; Klepzig et al., 1996; Rieske and Raffa, 1998). These impacts can be due to competition for a limited resource, especially when different species feed on the same plant tissue, even if they are temporally segregated (Denno et al., 1995). In cases where insect herbivores from distinctly separated plant feeding guilds exert interspecific effects, however, alterations in host physiology or morphology by the first herbivore appear to mediate the relationship between the second herbivore and its host (Strong, 1983; Faeth, 1986; Hunter and Price, 1992). Although such relationships are becoming increasingly well documented (Ericsson et al., 1980; Wood, 1982; Strong, 1983; Rhoades, 1985; Faeth, 1986, 1992a,b; Lewinsohn et al., 1991; Klepzig et al., 1996), the underlying chemical and physiological processes by which these alterations occur remain poorly understood (McNaughton, 1983; Krause and Raffa, 1996). Some of the proposed mechanisms include increases or decreases in host defensive capability (McNaughton, 1983; Krause and Raffa, 1996), changes in the nutritional quality of the feeding substrate (Scriber and Slansky, 1981; Rieske and Raffa, 1998), and reallocation of limited resources such as carbon (Lorio, 1986).

Defoliation of conifers can increase subsequent susceptibility to wood-boring insects and their symbiotic fungi (Wright et al., 1984; Raffa et al., 1998). Colonization by these subcortical species, when successful, is almost always fatal to the host (Rudinsky, 1962; Wood, 1972; Raffa and Berryman, 1983b).

Host conifer defenses against subcortical insects provide a useful model for evaluating herbivore mediation of subsequent herbivore–host interactions. These defenses include both preformed and induced components, each of which

is essential to the host (Berryman, 1972; Hain et al., 1983; Paine and Stephen, 1988; Paine et al., 1988; Raffa, 1991; Nebeker et al., 1992; Cates, 1996). Conifer phloem tissue contains monoterpenes and phenolics at constitutive concentrations that can have some deterrent action on bark beetles. Once the living tissue is attacked, the phloem tissue in the immediate area of the wound undergoes rapid histological changes, which can confine the invading complex. Within the resulting lesions, host allelochemicals can rise rapidly to repellent, insecticidal, and fungistatic concentrations. Bark beetles can collectively exhaust these metabolically costly responses, however, by engaging in mass attacks, which are mediated by aggregation pheromones (Wood, 1972; Borden, 1982; Seybold et al., 1995b). The preformed resin system of pines can delay beetles as they bore through the bark, and perhaps interfere with pheromone emission from beetle entry sites (Raffa and Berryman, 1983a), thereby allowing induced responses sufficient time to mobilize in advance of the attacking cohort. Evaluating chemical changes associated with defoliation provides an opportunity to evaluate the relative impacts of a single stress agent on both constitutive and induced mechanisms.

In a study involving a folivore, the jack pine budworm *Choristoneura pinus pinus* (Freeman) (Lepidoptera: Totricidae), feeding on jack pine *Pinus banksiana* (Lamb), the likelihood of subsequent colonization by the bark beetle *Ips grandicollis* and its associated fungus *Ophiostoma ips* and the wood borer *Monochamus carolinensis* rose exponentially with the degree of defoliation (Wallin, 1996). Feeding by *C. pinus pinus* alters both the preformed system, as measured by the rate of resin flow from a mechanical wound, and the induced system, as measured by the rate of lesion closure in response to challenge inoculation with *O. ips* (Wallin, 1996). The patterns of these changes are complex, ranging from a parabolic relationship between defoliation intensity and defensive parameters immediately after defoliation, to exponentially declining relationships across all defoliation intensities after one year. Colonization by subcortical insects and fungi and tree death closely followed the latter pattern. The photosynthetic rates of the remaining needles were not affected by defoliation, but tree growth rates were strongly and negatively impacted. Resin flow and induced responses followed similar trends.

The chemical changes within host subcortical tissue that accompany defoliation are not well known. In conifers, monoterpenes and phenolics are the principal chemical groups involved in defense against subcortical insects and pathogens (Raffa, 1991; Klepzig et al., 1996). Monoterpenes have a wide variety of functions in these interactions (Berryman, 1972; Coyne and Lott, 1976; Langenheim, 1994). For example, certain monoterpenes can serve as synergists or precursors of bark beetle pheromones or as attractants or feeding stimulants at low concentrations, thus facilitating beetle orientation towards its host (Vité et al., 1962; Hughes, 1974; Miller and Borden, 1990; Seybold et al., 1995a,b).

Conversely, other monoterpenes, or the same monoterpenes at higher concentrations, can repel or kill colonizing adults, prevent egg hatch, inhibit fungal spore germination, and inhibit fungal mycelial growth (Coyne and Lott, 1976; Raffa and Berryman, 1983b; Raffa et al., 1985; Langenheim, 1994; Klepzig et al., 1996). A number of studies have demonstrated that the composition and/or concentration of monoterpenes in either constitutive or inducible phloem tissue can be associated with subsequent likelihood of host mortality to subcortical organisms (Raffa and Berryman, 1982, 1983b; Nebeker et al., 1992).

This study reports the quantitative and compositional changes that occur in the phloem of jack pine following varying intensities of natural defoliation by *C. pinus pinus*. We quantified these changes in both components of the defensive system, constitutive and induced monoterpenes, in response to simulated attack by *I. grandicollis*, with *O. ips* as the challenge.

METHODS AND MATERIALS

Plot Description and Quantification of Natural Defoliation Level. Three plots of 66 trees each were established in a *P. banksiana* stand in Jackson County, Wisconsin (T.20N R2W sec. 17) in August 1994. Within each plot, the trees had been naturally defoliated to varying degrees by *C. pinus pinus* during the previous month. The plots are relatively homogeneous with regard to soil, slope, and ground cover, and selected trees were similar in size (4.5 ± 0.5 cm dbh) and phenotypic characteristics (overstory and dominant) (Wallin, 1996).

Defoliation levels were determined for each tree by averaging the independent estimates of three observers based on established criteria (Wisconsin and Michigan Departments of Natural Resources, T. Lanigan and D. McCullough, personal communication). The resultant categories are: (1) no visible defoliation, (2) ≤25% defoliation, (3) 26–50% defoliation, (4) 51–75% defoliation (no top kill present), and (5) >76% defoliation (top kill present). These classes are subsequently referred to as no, light, moderate, heavy, and severe defoliation levels. These visual defoliation categories were subsequently verified and quantified by destructively sampling randomly chosen subsets of trees within each class (Wallin, 1996).

Effects of Defoliation Levels on Constitutive and Induced Responses to Subcortical Insects and Fungi. Constitutive and induced monoterpene levels were measured in early June 1995, early August 1995, and early June 1996. Simulated attacks were induced by inoculation with *O. ips*. Host responses to such inoculations are chemically and histologically similar to those of natural single attacks (Raffa and Berryman, 1982).

O. ips was isolated in February 1995 from *P. banksiana* phloem tissue colonized by *I. grandicollis* at the field sites and cultured by methods described

in Raffa and Smalley (1988). These cultures were grown in Petri plates on potato dextrose agar at 22°C and 85% relative humidity. Following 7–10 days of growth, they provided the source for hyphal tip transfer. Three adjacent inoculations were administered evenly around the circumference of five randomly selected trees from each defoliation class at each sampling interval for a total of 75 trees. Each inoculation of *O. ips* was administered at 1.4 m into the main stem to simulate introduction by *I. grandicollis*. We administered inoculations by boring a hole to the sapwood with a 4-mm-diameter cork borer, applying a mycelial plug into the sapwood with a 3-mm-diameter metal syringe, and then resecuring the bark (Raffa and Berryman, 1982). Three, six, and nine days following inoculation, the outer bark and phloem tissue were removed from one of the three inoculations, thereby exposing fungal response lesions on the inner phloem and outer xylem. The visible reaction lesions on the surface of the phloem–xylem interface were measured and removed. The excised tissue was placed in a vial, immediately stored over Dry Ice in the field, and subsequently stored at −10°C until analysis.

The monoterpene quantity and composition of test trees were analyzed by gas–liquid chromatography, with the conditions of Raffa and Steffeck (1988). Phloem samples were finely chopped, and extracted in a solution of 10 ml pentane–0.1% *p*-cymene for 24 hr. *P*-Cymene serves as a qualitative internal standard, as it is not present in *P. banksiana* phloem and is easily separated from the naturally present monoterpenes. Extracts were separated from the phloem by vacuum filtration and dried over calcium chloride for approximately 1 hr. Concentrations of each monoterpene were determined by integrating peak areas with a Shimadzu GLC 9A (Shimadzu Scientific Instruments, Inc., Columbia, Maryland). Separations were performed on a 25-m × 0.25-mm bonded fused silica open tubular polyethylene glycol column (Alltech Assoc., Deerfield, Illinois). The oven temperature was 60°C for 10 min, increased to 160°C at 10°C/min. Helium flow at 30 cm/sec was the carrier gas. Methane had a retention time of 83.8 sec. The proportions of various monoterpenes were determined by integrating peak areas and comparing each peak with known standard equations. Standard equations were calculated by regressing the peak areas of pure monoterpenes from Alltech Assoc., Deerfield, Illinois, on known quantities (α-pinene R^2 = 0.997, β-pinene R^2 = 0.999, carene R^2 = 0.999, myrcene R^2 = 0.964, limonene R^2 = 0.994, terpinene R^2 = 0.999) (Wallin, 1996). Following analysis, each phloem sample was oven-dried and weighed. Total monoterpenes were determined on both a per gram of lesion tissue and a per total lesion basis.

Mean concentrations of individual and total monoterpenes were calculated and compared by repeated measures ANOVA within each month (SAS, 1989). Responses across months were also compared by treatment with split-plot ANOVA (SAS, 1989). Homogeneity of variance was tested with the Levenes test and heterogeneous variances were log transformed prior to analysis (SAS, 1989).

Effect of Host Phloem Monoterpenes on I. grandicollis *and* M. carolinensis *Incidence.* We quantified colonization of trees by bark beetles and wood borers across the various defoliation levels, by the method of Wallin (1996). Briefly, a 2 m^2 section of the bole of each tree ($N = 168$) was visually assessed, marked, and quantified for bark beetle holes and wood borer oviposition marks during March to November of each year (1994, 1995, and 1996). Each entrance hole and oviposition site was marked to designate time of attack. The counts were pooled into three periods, August 1994–June 1995, July 1995–October 1995, and April 1996–August 1996.

Totals of *I. grandicollis* and *M. carolinensis* entrance holes and oviposition marks, respectively, were separately regressed (SAS, 1989) against individual monoterpenes, total monoterpenes, and on the ratio of α-pinene to β-pinene based on the phytochemical changes we observed. Regressions were performed with results from constitutive tissue and tissue three, six, and nine days following inoculation with *O. ips*. Responses across defoliation levels were also regressed within constitutive tissue and within each day following inoculation. Non- and lightly defoliated trees were not included in the regression analysis, since trees at these defoliation levels were not colonized by *I. grandicollis* or *M. carolinensis* (Wallin, 1996). Heterogeneous variances were transformed prior to analyses. Means of individual and total monoterpenes were calculated for trees with and without insects present in June 1995. Survivorship of each tree was also correlated with *I. grandicollis* or *M. carolinensis* colonization by chi-square analysis (SAS, 1989).

RESULTS

Effects of C. pinus pinus *Feeding on Constitutive and Induced Monoterpenes in Response to Simulated Attacks by Subcortical Insect–Fungal Complexes.* The proportions of various monoterpenes in *P. banksiana* phloem varied between constitutive and induced tissue. In the overall model, there was a significant effect of the time after inoculation on the proportions of α-pinene ($F_{2,177} = 9.616$, $P < 0.001$), β-pinene ($F_{2,177} = 7.765$, $P < 0.0001$), myrcene ($F_{2,177} = 3.837$, $P < 0.0111$), limonene ($F_{2,177} = 14.65$, $P < 0.0001$), and terpinene ($F_{2,165} = 5.709$, $P < 0.001$). Proportions of monoterpenes also varied among defoliation intensities, with time after defoliation, and due to various interactions among these parameters (Table 1).

The monoterpene fraction of constitutive (day 0) phloem tissue was comprised almost entirely of α-pinene and β-pinene, with α-pinene being the most abundant (Table 2). The relative proportions of these compounds in constitutive tissue were significantly affected by defoliation intensity. These ratios averaged 10.45 in nondefoliated trees, 1.74 in lightly defoliated trees, 3.91 in moderately

TABLE 1. EFFECTS OF *C. pinus pinus* FEEDING ON PERCENTAGE OF MONOTERPENES IN PHLOEM REACTION TISSUE OF *Pinus banksiana*

Days after Inoculation	Source	*df*	α-Pinene		β-Pinene		Δ^3-Carene		Myrcene		Limonene		Terpinene	
			F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
0	Defoliation level (DL)	4,14	1.127	0.361	0.826	0.494	0.979	0.422	0.972	0.424				
	Time after defoliation (TD)	2,12	1.174	0.290	2.207	0.015	9.140	0.007	1.167	0.292				
	DL * TL	6,18	0.067	0.935	0.336	0.712	1.468	0.253	1.167	0.330				
3	DL	4,14	2.397	0.083	1.64	0.196	2.300	0.093	0.742	0.534	4.188	0.012	1.160	0.337
	TD	2,12	4.997	0.012	5.571	0.007	1.549	0.226	6.042	0.005	7.002	0.003	0.238	0.789
	DL * TL	6,18	3.417	0.012	3.637	0.008	0.580	0.715	1.114	0.369	1.405	0.245	0.471	0.795
6	DL	4,14	3.860	0.049	2.38	0.089	2.029	0.110	0.052	0.994	3.718	0.059	0.686	0.606
	TD	2,12	10.700	<0.001	25.89	<0.001	7.147	0.002	18.590	<0.001	2.616	0.087	1.801	0.179
	DL * TL	6,18	1.053	0.401	0.48	0.788	2.854	0.028	0.252	0.936	1.284	0.291	0.950	0.460
9	DL	4,14	1.936	0.126	0.883	0.484	3.209	0.024	6.507	0.000	3.052	0.030	0.216	0.927
	TD	2,12	15.380	<0.001	7.921	0.001	8.249	0.001	20.970	<0.001	3.149	0.056	41.780	<0.001
	DL * TL	6,18	3.013	0.023	1.494	0.217	4.807	0.002	10.350	<0.001	4.549	0.003	0.184	0.966

TABLE 2. EFFECTS OF *C. pinus pinus* FEEDING ON *P. banksiana* MONOTERPENE COMPOSITION OF CONSTITUTIVE AND INDUCED PHLOEM TISSUE[a]

		Days following inoculation											
		June 1995				August 1995				June 1996			
Defoliation level	Monoterpene (%)	0	3	6	9	0	3	6	9	0	3	6	9
None	α-Pinene	92.9 Aa	74.6 Ab	75.3 Ab	74.8 Ab	88.3 Aa	78.6 Aa	75.3 Aa	74.8 Aa	87.4 Aa	75.3 Ab	15.1 Ac	83.7 Ab
	β-Pinene	6.1 Aa	21.7 Ab	22.6 Ab	22.9 Ab	10.1 Aa	18.5 Ab	22.8 Ab	23.3 Ab	9.1 Aa	21.9 Ab	58.9 Ac	13.9 Ad
	Δ^3-Carene	1.0a	0.74 Ab	0.002 Ac	0.04 Ad	1.4a	0.38 Aa	0.02 Ab	0.009 Ab	3.4a	1.9 Ab	7.1 Ac	0.6 Ad
	Myrcene	0.0a	0.89 Ab	0.9 Ab	0.92 Ab	0.0a	0.87 Ab	0.62 Ab	0.76 Ab	0.0a	0.34 Ab	17.5 Ac	1.4 Ad
	Limonene	0.0a	1.7 Ab	0.6 Ac	0.7 Ac	0.0a	1.1 Ab	0.67 Ac	0.56 Ac	0.0a	0.22 Ab	0.69 Ac	0.24 Ab
	Terpinene	0.0a	0.32 Ab	0.64 Ab	0.64 Ab	0.0a	0.53 Ab	0.62 Ab	0.61 Ab	0.0a	0.37 Ab	0.74 Ac	0.0 Aa
Light	α-Pinene	54.5 Ba	67.4 Ab	74.5 Ac	69.4 Ad	64.4 Ba	77.7 Ab	83.2 Ac	73.9 Ab	58.5 Ba	81.6 Ab	16.2 Ac	73.5 Ad
	β-Pinene	45.5 Ba	28.8 Ab	23.4 Ac	28.1 Bb	35.6 Ba	19.5 Ab	13.6 Bc	24.1 Ab	40.5 Ba	13.7 Ab	64.0 Bc	24.5Bd
	Δ^3-Carene	0.0a	0.41 Bb	0.004 Ac	0.005 Bc	0.0a	0.35 Ab	0.006 Ac	0.006 Ac	1.4a	0.76 Ab	0.92 Bb	0.85 Bb
	Myrcene	0.0a	1.3 Ab	0.9 Ab	1.03 Ab	0.0a	0.95 Ab	1.64 Bc	0.84 Ab	0.0a	0.62 Ab	16.6 Ac	0.63 Ab
	Limonene	0.0a	1.4 Ab	0.63 Ab	0.63 Bb	0.0a	0.79 Ab	0.79 Ab	0.54 Ab	0.0a	0 Ba	0.96 Bb	0.55 Ac
	Terpinene	0.0a	0.82 Ab	0.58 Ac	0.78 Ab	0.0a	0.7 Ab	0.4 Ac	0.58 Ad	0.0a	3.3 Ab	1.3 Ac	0.0 Aa
Moderate	α-Pinene	82.1 Ca	67.1 Ab	75.3 Ab	72.7 Ab	76.4 Ca	81.4 Aa	79.1 Aa	79.6 Aa	79.7 Aa	85.4 Ba	32.6 Ab	16.2 Bc
	β-Pinene	16.5 Ca	27.5 Ab	22.3 Ac	25 Ab	23.7 Ca	15.9 Aa	18.8 Aa	18.4 Ba	20.3 Ca	13.4 Ab	45.3Cc	63.7Cd
	Δ^3-Carene	0.0a	1.1 Ab	0.002 Ac	0.006 Bc	0.0a	0.42 Ab	0.005 Aa	0.0 Ba	0.0a	0.82 Ab	0.6Cb	0.75 Cb
	Myrcene	1.4a	1.1 Ab	1.09 Bb	1.01 Ab	0.0a	0.76 Ab	0.65 Ab	0.72 Ab	0.0a	0.21 Ab	20.6 Ab	18.3 Bb
	Limonene	0.0a	1.9 Ab	0.65 Ac	0.64 Bc	0.0a	1.1 Ab	0.86 Ab	0.37 Bc	0.0a	0.03 Aa	0.57 Cb	0.94 Bc
	Terpinene	0.0a	1.4 Ab	0.59 Ac	0.66 Ac	0.0a	0.46 Ab	0.47 Ab	0.56 Ab	0.0a	0.14 Ab	0.37 Ab	0.0 Aa
Heavy	α-Pinene	80.8 Ca	74.3 Ab	70.7 Ab	69.3 Ab	75.7 Ca	78.6 Aa	80.4 Ab	70.1 Ac				
	β-Pinene	19.2 Ca	20.2 Aa	26.9 Bb	27.9 Bb	24.3 Ca	18.3 Ab	17.1 Ab	27.9 Aa				
	Δ^3-Carene	0.0a	1.1 Ab	0.01 Bc	0.03 Ac	0.0a	0.4 Ab	0.32 Bb	0.0 Ba				
	Myrcene	0.0a	0.83 Ab	0.98 Ab	1.05 Ab	0.0a	0.62 Ab	0.64 Ab	0.72 Ab				
	Limonene	0.0a	2.7 Ab	0.61 Ac	0.69 Ac	0.0a	1.24 Ab	1.2 Bb	0.64 Ac				
	Terpinene	0.0a	0.8 Ab	0.72 Ab	0.75 Ab	0.0a	0.68 Ab	0.44 Ac	0.63 Ab				
Severe	α-Pinene			71.3 Aa	75.9 Ab								
	β-Pinene			26.5 Aa	22.3 Ab								
	Δ^3-Carene			0.0 Ca	0.0 Ca								
	Myrcene			0.84 Aa	0.69 Ab								
	Limonene			0.59 Aa	0.51 Ca								
	Terpinene			0.7 Aa	0.56 Ab								

[a]Within each month, means within a row followed by the same lowercase letter are not significantly different; within each column, means of separate monoterpenes followed by the same uppercase letter are not significant ($P < 0.05$).

defoliated trees, and 3.67 in heavily defoliated trees. The ratio of α-pinene to β-pinene in constitutive tissue was always highest in the nondefoliated trees. This altered ratio in constitutive phloem tissue in response to defoliation was consistent both across the three sampling periods, and across all levels of defoliation.

Induced tissues showed a higher diversity of monoterpenes than did constitutive tissues and were comprised of α-pinene, β-pinene, myrcene, limonene, 3-carene, and terpinene (Table 2). These additional compounds appeared within three days after inoculation in all cases. As in constitutive tissue, α-pinene and β-pinene were the most abundant monoterpenes in phloem reaction lesions, regardless of treatment or time since defoliation. Proportionate changes in the monoterpene fraction occurred within three days in response to challenge inoculation, but these changes did not reflect effects of defoliation until several days later (Table 1). Because the proportions of monoterpenes three days after controlled inoculation with *O. ips* did not vary among defoliation levels, regardless of the time after defoliation, they are not considered further. Conversely, from three to six days following inoculation, proportionate changes in α-pinene, β-pinene, 3-carene, myrcene, limonene, and terpinene were associated with defoliation intensity, time since defoliation, and various interactions.

Proportional changes in monoterpenes during days 6–9 following challenge inoculation varied among months and defoliation levels (Table 2). In June 1995, carene (1.038%) increased in nondefoliated trees. During induction, proportions of β-pinene (4.7%), myrcene (0.13%), and terpinene (0.2%) increased, and α-pinene (5.1%) decreased in lightly defoliated trees. β-Pinene (2.7%) also increased in trees that were moderately defoliated. The proportions of α-pinene (6.6%) increased, and proportions of β-pinene (4.2%), myrcene (0.15%), and terpinene (0.14%) decreased in severely defoliated trees. In August 1995, β-pinene (10.5%) and terpinene (0.18%) increased in lightly defoliated trees. α-Pinene (9.3%) and myrcene (0.8%) decreased in lightly defoliated trees. Limonene (0.49%) decreased in moderately defoliated trees. β-Pinene (9.8%) and terpinene (0.19%) increased and α-pinene (10.3%), carene (0.32%), and limonene (0.56%) decreased in heavily defoliated in trees. In June 1996, α-pinene (68.8%) increased and β-pinene (40.5%), carene (6.5%), myrcene (16.1%), limonene (0.45%) and terpinene (0.74%) decreased in nondefoliated trees. The proportions of α-pinene (57.3%) increased and β-pinene (39.5%), myrcene (15.97%), limonene (0.41%), and terpinene (1.3%) decreased in lightly defoliated trees. β-Pinene (18.4%) and limonene (0.37%) increased and α-pinene (16.4%) decreased in moderately defoliated trees.

Because the changes in various monoterpene compositions in response to simulated attack by *I. grandicollis–O. ips* associations were complex, we have summarized the data by pooling all monoterpenes within treatments in Table 3. For each defoliation level, time after inoculation, and time after defoliation, the

TABLE 3. SUMMARY OF PROPORTIONATE CHANGES AMONG MONOTERPENES IN *P. banksiana* FOLLOWING CHALLENGE INOCULATION WITH *O.ips*[a]

	Percentage of monoterpenes that changed in each direction		
	Decrease	Increase	No Change
Defoliation level			
None	18.5	42.5	39.1
Light	33.4	48.1	18.2
Moderate	20.1	33.4	46.6
Heavy	22.2	33.3	44.5
Days after inoculation			
0–3	13.6	77.4	9.0
3–6	31.8	35.8	42.4
0–6	13.6	80.4	6.0
6–9	29.1	16.6	54.3
0–9	15.0	65.0	20.0
Time since defoliation			
June 1995	23.0	35.9	41.1
August 1995	20.8	34.7	44.5
June 1996	33.4	50.0	16.6

[a]Values show the percentage of monoterpenes in each category that underwent statistically significant ($P < 0.05$) decreases, or increases, or showed no difference between constitutive and reaction tissue.

percentage of monoterpenes in *P. banksiana* phloem that significantly decreased, increased, or stayed the same in response to controlled inoculations is shown. In general, trees that were not or lightly defoliated had more monoterpenes that increased following challenge inoculation, than did moderately or heavily defoliated trees (Table 3). On average, host tree responses were quite rapid, and most monoterpenes showed significant increases during the first three days of induction (Table 3). The time after defoliation did not affect overall trends in any consistent manner (Table 3).

In addition to the above proportionate changes, there were also significant changes in the total quantities of phloem monoterpenes that were associated with both challenge fungal inoculation and budworm defoliation (Table 4). Total monoterpene contents of reaction tissue were much higher than those in constitutive phloem tissue (Table 5). Defoliation affected the total monoterpene concentration of induced reaction, but not constitutive tissue. In constitutive phloem tissue, total monoterpene concentrations did not vary among defoliation levels during any period after defoliation (Table 4). However, the abilities of trees to undergo induced reactions varied significantly among defoliation levels. These differences were manifested in both the total accumulation, and the rate of accu-

TABLE 4. REPEATED-MEASURES ANOVA OF TOTAL MONOTERPENES FOUND IN PHLOEM REACTION LESION OF DEFOLIATED *P. banksiana* FOLLOWING CHALLENGE INOCULATION WITH *O. ips*

Days after inoculation	Source	*df*	Total monoterpenes			
			mg/g		mg/lesion	
			F	*P*	*F*	*P*
0	Defoliation level (DL)	4	1.76	0.2		
	Time after defoliation (TD)	2	1.142	0.304		
	DL × TD	6	0.983	0.3901		
3	DL	4	2.079	0.1197	3.013	0.082
	TD	2	3.922	0.0285	2.979	0.0614
	DL × TD	6	3.24	0.0159	2.209	0.0708
6	DL	4	4.386	0.0052	2.392	0.0658
	TD	2	5.533	0.0078	4.564	0.0161
	DL × TD	6	4.242	0.0032	0.822	0.5408
9	DL	4	1.679	0.1175	2.53	0.0534
	TD	2	6.869	0.0031	3.776	0.0305
	DL × TD	6	1.172	0.3398	0.779	0.5905

mulation of monoterpenes. In general, lightly and moderately defoliated trees had greater monoterpene concentrations or reached their greatest monoterpene concentration more quickly, than did heavily defoliated trees. For example, by day 6, total monoterpene concentration in induced phloem tissue was highest in non-, lightly, or moderately defoliated trees (Table 5A). By day 9, this effect was often obscured or even reversed. Time since defoliation compounded these effects. For example, in June 1995, total monoterpene concentration increased from days 3 to 9 in each defoliation level (Table 5A). Conversely, in August 1995, total monoterpenes increased from days 6 to day 9 in trees that were heavily defoliated, but reached their maximum concentration within 6 days among trees that were either not or lightly defoliated.

When total monoterpenes are considered on a per lesion basis, a similar but slightly different pattern emerges. Total monoterpenes per lesion showed significant variation among defoliation intensities, and these differences appeared at each post-inoculation sampling interval (Table 5B). Total monoterpenes per lesion were less sensitive to the time interval since defoliation than were total monoterpenes per gram of phloem. In general, the induced reactions of more heavily defoliated trees were slower or less pronounced than those of non- or partially defoliated trees (Table 5B). Lightly and moderately defoliated trees often had higher quantities of accumulated monoterpenes following challenge

TABLE 5. EFFECTS OF *C. pinus pinus* FEEDING ON TOTAL MONOTERPENES IN *P. banksiana* PHLOEM[a]

Defoliation Level	Days since inoculation: June, 1995				August, 1995				June, 1996			
	0	3	6	9	0	3	6	9	0	3	6	9
A. Total monoterpene content (mg/g phloem)												
None	1.07 Aa	48.19 Ab	158.43 Ac	137.37 Ac	0.89 Aa	136.87 Ab	293.71 Ac	152.27 Ad	1.08 Aa	37.05 Ab	218.58 Ac	249.70 Ac
Light	1.10 Aa	54.30 Ab	86.35 Bb	174.51 Ac	0.86 Aa	99.39 Ab	328.12 Ac	102.38 Bb	1.10 Aa	4.48 Ba	67.59 Bb	205.32 Ac
Moderate	2.49 Aa	52.02 Ab	82.45 Bb	193.00 Ac	1.37 Aa	72.30 Ab	98.43 Bb	89.46 Bb	2.49 Aa	68.48 Cb	86.63 Bb	238.82 Ac
Heavy	2.23 Aa	49.15 Ab	132.89 Bc	113.47 Ad	1.44 Aa	54.44 Ab	71.79 Bc	117.02 Cd				
Severe	0.00 Aa	0.00	110.57 Bb	207.35 Bc								
B. Total monoterpene content (mg/g phloem)												
None	0.00	299.30 Aa	415.80 Aa	3152.20 Ab	0.00	485.40 Aa	178.22 Aa	3575.20 Ab	0.00	182.00 Aa	1497.40 Ab	3223.2 Ac
Light	0.00	394.20 Aa	2669.20 Bb	4522.70 Bc	0.00	469.00 Aa	314.04 Ba	2363.90 Bb	0.00	55.21 Ba	1073.90 Ab	56.36 Ba
Moderate	0.00	283.70 Aa	2643.20 Bb	5837.10 Cc	0.00	144.51 Ba	73.88 Cb	1362.30 Cb	0.00	248.53 Ca	823.30 Ab	3341.40 Ac
Heavy	0.00	617.10 Ba	2618.50 Bb	3560.00 Ab	0.00	304.52 Aa	88.26 Cb	1577.80 Cc				
Severe	0.00	0.00 Ca	984.00 Cb	1161.11 Db								

[a]Within each month, days followed by the same lowercase letter are not significantly different. Within each month, defoliation levels followed by the same uppercase letter are not significantly different.

TABLE 6. REGRESSION DATA FOR *I. grandicollis* COLONIZATION OF *P. banksiana* AS A FUNCTION OF TISSUE MONOTERPENES AFTER CHALLENGE INOCULATION WITH *O. ips* ($N = 13$)

Days after Inoculation	Monoterpene	R^2	P	Intercept	Slope
0	α-Pinene/β-pinene	0.886	<0.001	0.138	−0.122
3	β-Pinene	0.291	0.050	−0.215	0.002
	Carene	0.637	<0.001	−0.174	0.003
	Limonene	0.325	0.042	3.393	−0.006
	Terpinene	0.411	0.018	−0.431	0.003
6	Carene	0.307	0.049	0.395	0.050
	Myrcene	0.364	0.029	0.112	0.004
9	β-Pinene	0.734	<0.001	6.612	−9.219
	Myrcene	0.321	0.044	0.331	316.671
	Limonene	0.376	0.026	0.115	12.309
	α-Pinene/β-pinene	0.404	0.020	3.988	−1.131

inoculations. Total monoterpene content per reaction lesion increased from days 3 to 9 with increased defoliation, regardless of time since defoliation. Differences among defoliation levels in the total quantities of monoterpenes per lesion increased with the length of time after defoliation. For example, in June 1995, the quantity of monoterpenes per lesion was not directly related to defoliation intensity, but in June 1996 and August 1996, total monoterpenes per reaction lesion tissue were lower as defoliation intensity increased.

Relationship of Subcortical Insect Colonization to Phloem Monoterpenes. The incidence of *I. grandicollis* was related to the proportions of several monoterpenes in *P. banksiana* phloem (Table 6). Colonization by *I. grandicollis* was negatively related to the ratio of α-pinene to β-pinene in the preattack host tissue. Colonization by *I. grandicollis* was also significantly related to the proportions of several monoterpenes in induced reaction host tissue (Table 6). As with constitutive tissue, the ratio of α-pinene to β-pinene was inversely related to *I. grandicollis* arrival rates in tissues nine days after inoculation. Colonization densities by *I. grandicollis* were positively related to Δ^3-carene (three and six days after challenge inoculation), myrcene (six and nine days after inoculation), and terpinene (three days). Of these, the greatest slope was generated with myrcene. β-Pinene and limonene showed statistically significant relationships with *I. grandicollis* density but were inconsistent, in that they generated either positive or negative slopes depending on the time since challenge inoculation.

As a class, trees that were colonized by *I. grandicollis* had lower proportions of α-pinene and higher proportions of β-pinene than did noncolonized trees (Table 7). Similar differences occurred between the trees that were dead versus alive at the end of this study (Wallin, 1996).

TABLE 7. *P. banksiana* MONOTERPENES (%) IN TREES COLONIZED ($N = 24$) AND NOT COLONIZED ($N = 36$) BY *I. grandicollis* FOLLOWING DEFOLIATION BY *C. pinus pinus* (STANDARD ERROR OF MEANS)

	I. grandicollis	
Monoterpene	Colonized	Not Colonized
α-Pinene	59.8 (0.055)	67.9 (0.031)
β-Pinene	40.1 (0.124)	31.9 (0.07)
Carene	0.017 (0.015)	0.019 (0.01)
Myrcene	0.062 (0.025)	0.031 (0.012)
Limonene	0.011 (0.002)	0.01 (0.002)
Terpinene	0.011 (0.003)	0.006 (0.428)

Oviposition by *M. carolinensis* was not related to the relative proportions of any monoterpenes. Moreover, *M. carolinensis* oviposition marks in relation to the proportions of monoterpenes did not vary among defoliation levels, regardless of the time after inoculation, or defoliation.

DISCUSSION

The monoterpene content and composition of *P. banksiana* phloem, and biochemical responses to simulated bark beetle attack were influenced by defoliation intensity, host seasonal phenology, and time since defoliation. Both constitutive and induced monoterpenes were impacted by defoliation, but not to equal extents. These chemical changes were accompanied by several physiological responses, such as reduced growth, increased mortality, and variable but usually reduced fungal confinement rates and resin volume (Wallin, 1996). Changes in host responses throughout the growing season were pronounced and may relate to phenological changes in host physiological requirements and allocation patterns (Mooney, 1972; Chapin et al., 1990; Lorio, 1993).

Total monoterpene concentrations were higher in induced than constitutive tissue. Four monoterpenes, α-pinene, β-pinene, myrcene, and carene, comprised a larger fraction of total monoterpenes in induced than constitutive tissue, regardless of defoliation level or time since defoliation. Changes in these compounds appear to represent part of an induced defense, as they are known to reduce the success of *Ips* and *Ophiostoma* species at high concentrations present in reaction tissues (Klepzig et al., 1996). The relative proportions of these compounds that are present during rapidly induced responses are affected by defoliation level and time since defoliation.

The rapid increase in monoterpenes in the more vigorous trees may lessen the opportunity for successful insect colonization (Berryman, 1972). The rela-

tionships among monoterpene concentrations and *I. grandicollis* arrival are complex, but appear to reflect what is known about their behavioral effects. For example, there was a positive relationship between myrcene concentration and *I. grandicollis* arrival. Myrcene is known to increase *Ips* aggregation by functioning as a synergist and/or precursor of their aggregation pheromones (Vité et al., 1972; Hughes, 1973; Seybold et al., 1995a). Secondly, bark beetle feeding rates were more strongly associated with the chemical composition of constitutive than reaction phloem chemistry. Although this pattern differs from the well-documented role of constitutive antifeedants in many plant–herbivore systems, and may even seem counter intuitive, it demonstrates the importance of rapidly changing rate dynamics in conifer–bark beetle interactions. Similar results were obtained by Klepzig et al. (1996), who reported that *I. pini* differentiated between root-diseased and healthy *Pinus resinosa* primarily on the basis of rapidly induced responses, and by Raffa and Berryman (1983b), who reported that attack abandonment by *Dendroctonus ponderosae* is elicited largely by the early stages of induced reactions in *P. contorta*. The current results suggest that chemical changes associated with active host responses may affect beetle behavior within only three days after attack. Among the changes in monoterpene chemistry, the ratio of α-pinene to β-pinene was most consistently associated with both defoliation by *C. pinus pinus* and susceptibility to *I. grandicollis*.

When considering how host tree chemistry could interact with bark beetle pheromones to influence overall attraction, foliar chemistry should be considered in addition to phloem chemistry (Borden, 1982; Hayes and Strom, 1994). In this system, foliar monoterpene composition is markedly different from phloem monoterpenes. Whereas phloem constitutive tissue is comprised almost entirely of α-pinene and β-pinene, foliage contains higher quantities of myrcene and carene than α-pinene, and substantial quantities of limonene (Wallin and Raffa, 1998). These tissues also differ in that phloem monoterpenes change in response to defoliation, whereas foliar monoterpenes remain stable. Thus, injury to one plant tissue not only can cause chemical changes in other tissues, but such changes can even be greater in the tissue not directly affected.

The influence of defoliation intensity on monoterpene accumulation suggests a link between production and allocation of carbon within *P. banksiana*. For example, monoterpenes are metabolically expensive to synthesize and store (Gershenzon, 1994; Langenheim, 1994). The reduction of total constitutive monoterpenes in defoliated trees without a significant reduction in growth in June 1995 (Wallin, 1996) lends support to growth-differentiation hypotheses (Lorio, 1986; Herms and Mattson, 1992; Koricheva et al., 1998). However, results from subsequent sampling periods showed a strong and consistent inverse relationship between defoliation level and induced monoterpene concentrations and growth, which agrees with plant stress theory (Waring and Pitman, 1983; Waring, 1987; Koricheva et al., 1988). Thus, no

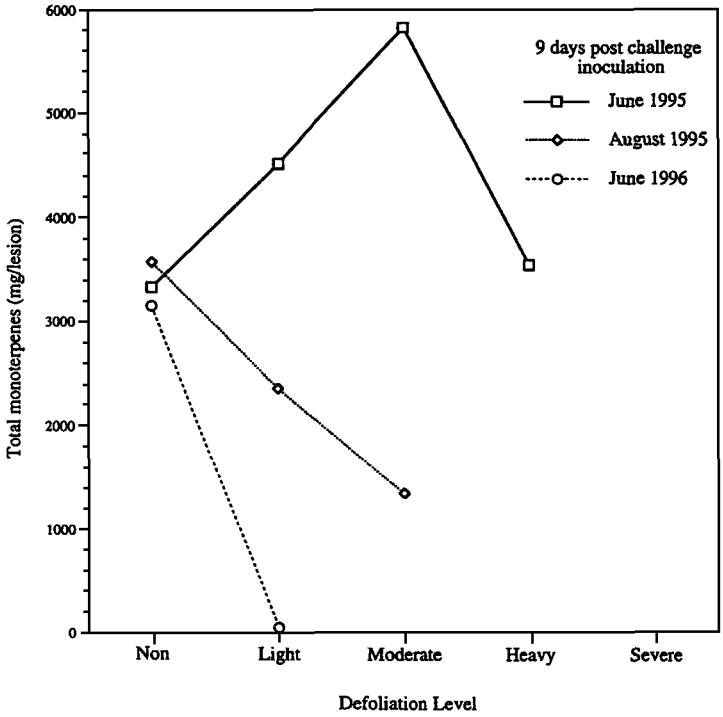

FIG. 1. Interacting effects of defoliation level and time since defoliation on induced accumulation of monoterpenes in *P. banksiana* phloem tissue.

one plant defense theory can totally explain the relationship between defoliation and subsequent susceptibility to subcortical insect–fungal complexes in *P. banksiana*. Rather, the duration of stress appears to be an important factor that can alter the relationship between stress and secondary chemistry profiles. This is illustrated in Figure 1. A major emphasis of allocation theories is that relationships between plant secondary chemistry and stress are not linear. However, our results also suggest that when time is included as a variant, parabolic versus direct decline relationships with stress may represent transitions along a continuum (Wallin, 1996). There may be a physiological basis for this transition. For example, the concentrations of carbon-based defenses in June 1995 were not consistently reduced by defoliation intensity, perhaps because defoliation occurred following the 1994 growing season. Trees that were severely defoliated eventually had the greatest total amount of induced monoterpenes on a per milligram basis, although they accumulated more slowly. By August 1995, however, the reduction of total monoterpenes in the induced subcortical reaction

tissue of defoliated trees apparently reflected the high cost of monoterpene production. These results further support the need for integration of theories such as growth-differentiation and plant stress (Herms and Raffa, 1995).

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REEVALUATION OF THE ROLE OF MANDIBULAR GLANDS IN REGULATION OF REPRODUCTION IN BUMBLEBEE COLONIES

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Abstract—Possible pheromonal control of worker reproduction was tested in *Bombus terrestris*. The mode of assay included exposure of callow workers to extracts originating from different queen parts and measuring the effect on the in vitro biosynthesis of juvenile hormone (JH), the apparent gonadotropin in this species. Both queen total body extracts applied to dummies consisting of oven-dried or Soxhlet-washed virgin queen bodies and cuticular washes applied to living virgin queens effectively inhibited the biosynthesis of JH in callow workers. None of the five exocrine glands (mandibular, hypopharyngeal, salivary, Dufour's, and tarsal) demonstrated inhibitory activity. Likewise, the use of synthetic 3-hydroxy acids, found in queen mandibular glands, were ineffective in blocking JH biosynthesis in queenless workers. The results suggest that the queen may use a primer pheromone spread on the epicuticle as a means to inhibit worker reproduction. However, our results are not consistent with the prevailing hypothesis that in *B. terrestris* the main source of the pheromone that inhibits worker reproduction is in the queen's mandibular glands.

Key Words—Bumblebee, communication, corpora allata, hydroxy acid, juvenile hormone, mandibular gland, primer pheromone, regulation of reproduction, behavior.

INTRODUCTION

Social insects have highly developed communication systems that enable them to regulate and coordinate complicated social processes. Pheromones serve as

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the main route for social communication and can be categorized into two types: "releaser" pheromones, eliciting a fast behavioral response; and "primer" pheromones, mediating prolonged physiological changes before evoking an eventual behavioral response. Releaser pheromones include chemicals used for sexual attraction, aggregation, alarm, trail marking, and recognition. The distinct and usually "all-or-none" behavioral response to these pheromones enables a relatively simple and short identification procedure. Indeed, the chemical composition of many releaser pheromones has been identified in a multitude of social Hymenoptera, revealing a plethora of chemical structures (Blum and Brand, 1972; Free, 1987; Wheeler and Duffieled, 1988; Hölldobler and Wilson, 1990). On the other hand, only a few primer pheromones have been chemically identified (Dulka et al., 1987; Slessor et al., 1988; Arnold et al., 1994). This seemingly poor achievement can be attributed to the prolonged and complicated processes mediated by primer pheromones, such as the regulation of reproduction and caste determination. Accordingly, the need to develop a long-term and complex bioassay has considerably hampered their identification.

In highly social insects with colonies too large for effective behavioral control, it is commonly believed that queen primer pheromones mediate regulation of reproduction (reviewed in Wilson, 1971; Michener, 1974; Hölldobler and Bartz, 1985; Fletcher and Ross, 1985; Spradbery, 1991). This hypothesis relies exclusively on indirect evidence, including observations on species in which worker reproduction is inhibited without any apparent behavioral control; inhibition of reproduction in workers that were separated from the queen by a single or a double mesh; and experiments in which worker reproduction was inhibited in the presence of dead queens or queen extracts (reviewed in Hölldobler and Bartz, 1985; Fletcher and Ross, 1985; Free, 1987: Hölldobler and Wilson, 1990; Spradbery, 1991). However, all efforts to isolate and chemically identify such queen pheromones have failed. Inhibition of worker reproduction could not be confirmed even for the well characterized honeybee mandibular gland queen substance (Butler, 1959; Butler et al., 1961). The complete identification of the chemical composition of the queen honeybee mandibular pheromone (Slessor et al., 1988) confirmed that it has several releaser effects on workers, e.g., inhibition of swarming (Winston et al., 1991) and enhancing worker foraging (Currie et al., 1994). However, its function as a primer pheromone that inhibits worker reproduction was refuted (Willis et al., 1990), leaving only the possibility of primer effect on queen rearing (Pettis et al., 1995; Slessor et al., 1998).

The multiple failures to show a causative effect of a queen pheromone in the social insects led to the development of an alternative hypothesis: namely, "queen signal and not queen control." According to this hypothesis, queen presence is conveyed by a queen signal, and the workers behave so as to maximize their inclusive fitness (Keller and Nonacs, 1993). This hypothesis emphasizes the ultimate mech-

anism for the evolution of worker sterility, but leaves an open question regarding the nature of the queen signals. In such cases queen presence may be conveyed by a complex set of signals that encompasses several communicative channels. For example, recent findings demonstrated that the queen honeybee may produce a queen specific signal in head glands other than the mandibular glands (Slessor et al., 1998). In this study we reevaluated the reported role of the queen bumblebee mandibular gland secretion in inducing worker sterility.

Colonies of the bumblebee *Bombus terrestris* are characterized by two distinct phases of regulation of reproduction. During the first phase, the queen is the only reproductively active female, while the following competition phase (CPh) is characterized by reproductive competition between the queen and dominant egg laying workers (Doorn and Heringa, 1986; Duchateau and Velthuis, 1988; Röseler and Honk, 1990). At least before the competition phase, worker reproduction is inhibited by the presence of the queen (Röseler, 1977; Röseler and Röseler, 1978; Duchateau and Velthuis, 1989; Bloch et al., 1996). One mechanism for inhibiting worker reproduction is to slow their ovarian development by suppressing the production of juvenile hormone (JH) which is apparently involved in the regulation of ovarian development in *B. terrestris*. Titers of JH III, the only JH homolog found in *B. terrestris* hemolymph, are correlated with ovarian development (Bloch et al., in preparation). Moreover, injection of JH to callow queenright workers resulted in dose-dependent ovarian development (Röseler, 1977). Several lines of evidence have suggested that inhibition of worker ovarian development is mediated by a primer pheromone secreted from the queen mandibular glands. Workers from colonies headed by queens whose mandibular glands had been surgically removed started to reproduce earlier than workers from colonies with intact, or sham-operated, queens (Honk et al., 1980). Corpora allata (CA, the glands that synthesize JH) volumes and in vitro biosyntheses of JH were lower in callow workers exposed to dead queen bodies, whole queens, or mandibular glands extracts (Röseler et al., 1981). Since in these studies queen mandibular gland extracts or cuticular washes had similar inhibitory effects to those of living queens, it was suggested that the putative queen pheromone is produced in these glands and then spread over the queen body by self-grooming (Röseler et al., 1981; Röseler and Honk, 1990). Notwithstanding, the radiochemical assay used for the measurement of JH biosynthesis in these studies was not optimized specifically for *B. terrestris*, and CA volume is not considered to be a reliable indication for their activity (Feyereisen, 1985). In some cases the statistical analysis may have been inadequate. Moreover, queens of *B. impatiens* from which the mandibular glands were surgically removed retained their full reproductive dominance (Pomeroy 1981, as cited in Plowright and Laverty, 1984).

In this article we reexamine the importance of exocrine secretions produced by various glandular sources in the regulation of reproduction in *B. terrestris*.

The parameter used in this study was the rate of JH biosynthesis in vitro in workers exposed to different queen-derived secretions, using a radiochemical assay (RCA), validated specifically for adult *B. terrestris* (Bloch et al., 1996).

METHODS AND MATERIALS

Bees. Colonies of *B. terrestris* were obtained from Bio-Bee Sde-Eliahu Industry, Bet Shean, Israel, a few days after the first worker had emerged. The colonies contained a queen, 1–10 workers, and brood at different stages of development. In the laboratory they were reared in Styrofoam nesting boxes (18 × 27 × 12 cm) with a cardboard bottom in an environmental chamber at a temperature of 29 ± 2°C and in constant darkness, except for a few minutes of light during the daily treatment. Commercial sugar syrup (Bee Happy) and fresh pollen (collected by honeybees) were supplied to all colonies ad libidum. Observations were made under dim red light through a glass lid placed on top of the nesting box.

Initiation of the competition phase (CPh) was defined by one or more of the following events: (1) the first day a worker was seen to oviposit; (2) two or more open egg cups were observed over two or more successive days; (3) only one open egg cup was observed over two or more successive days, but there were clear signs of cup destruction; and/or (4) oophagy was observed (Bloch et al., 1996).

Extract Preparation. Extracts were made from queens collected from normally developing colonies either before or during the CPh. The queens were killed by freezing (30–60 min) at −20°C, and were either extracted immediately or stored at −80°C until extraction. Total body extracts were prepared by cutting the bees into small pieces and immersing them in a glass scintillation vial (20 ml) filled with the extraction solvent. Two different extraction procedures were used: (1) 24 hr with pentane (Merck) followed by an additional 10–12 hr in absolute ethanol (Riedel de Häen) (pentane–ethanol extract). The pentane and ethanol extracts were applied separately, one following the other on the dummies before the bioassay; and (2) three successive extraction periods, each for 2 hr, in chloroform–methanol 2 : 1 (Blight and Dyre, 1959) (chloroform–methanol extract). Cuticular washes were obtained by extracting frozen queens to reduce extraction of fat body and internal organs (Gilby, 1980), for 5 min in chloroform–methanol (2 : 1). Extracts were first concentrated in a vacuum evaporator and then under nitrogen gas flow.

For glandular extraction, queens were dissected under chilled water, and the glands were transferred into 1.5 ml chloroform–methanol (2 : 1). The following exocrine glands were extracted: mandibular (MG), hypopharyngeal (HPG), head salivary (SG), Dufour's (DG), and tarsal (TG). The cephalic glands (MG, HPG, and SG) were removed in sequence to prevent cross contamination during the dissections.

JH Biosynthesis Rates. To measure rates of JH biosynthesis, we used an in vitro radiochemical assay (RCA) (Pratt and Tobe, 1974; Tobe and Pratt, 1974) especially optimized for adult *B. terrestris* (Bloch et al., 1996). Briefly, the bees' heads were immersed in modified bee saline under a dissecting microscope and the corpora allata–corpora cardiaca complex was cleaned of any adhering tissue and removed through an opening in the ventral part of the head. The glands were transferred to borosilicate tubes (6 × 50 mm) containing 25 μl of methionine-free bee medium. No more than 2 hr elapsed from the first dissection to the initiation of incubation. Incubation was started by adding an additional 25 μl of bee medium containing L-[^{3}H-methyl]methionine (NEN, specific activity 200 mCi/mmol). The glands were incubated for 3 hr at 39°C. At the end of the incubation the glands were removed, and 100 μl of double distilled water were added to the medium that was subsequently extracted following the method of Feyereisen and Tobe (1981).

Dummy Preparation. In most of the experiments, dead virgin queens were used as dummies for extract application. They were prepared by two different methods: (1) after emptying the gaster the queens were dried for one to two weeks in a vacuum oven at 45°C (oven-dried dummies); or (2) washing for 7–12 hr in a Soxhlet distillator with 2 : 1 chloroform–methanol as solvent (Soxhlet-washed dummies). The effects of queen cuticular washes and mandibular gland extracts were also examined by treating live callow virgin queens collected on the day of emergence, still before yellow pigmentation. In these experiments, the chloroform–methanol extracts were evaporated to dryness and then reconstituted in pentane. Following application the queens were introduced into the queenless group. The introduced queens were replaced once a day to prevent their possible integration into the group social hierarchy (Bloch et al., 1996). In two experiments we tested the effect of queen mandibular gland extracts after application on a glass slide or a Petri dish covered with melted honeybee wax.

Bioassay. Dummies were treated with one queen equivalent (Qeq; in 50–150 μl of solvent on dead dummies, or 20–50 μl on living queens), or solvent in the control groups. After letting the solvent evaporate they were housed with three callow workers that were less than 12 hr after emergence and before full coloration, in an experimental cage (107 × 107 × 95 mm). To reduce intergroup genetic variation, the workers were selected randomly from a pool of callow workers originating from different colonies. Dummies were replaced every day by freshly treated ones. After three days, or unless otherwise specified, the callow bees were sacrificed and analyzed for in vitro JH biosynthesis rates.

The biological activity of the hydroxy acid constituents of the mandibular glands (Hefetz et al., 1996) was tested using a blend containing 9 μg of 3-hydroxyoctanoic acid (3-HOA), 15 μg of 3-hydroxydecanoic acid (3-HDA), and 1.5 μg of 3-hydroxydodecanoic acid (3-HDDA) in methylene chloride. Thirty microliters of the solution, approximately equivalent to the quantity found in the

mandibular glands of six queens (6 Qeq), were applied to an oven-dried dummy and a pea-size (approximately 5-mm diameter) wax ball. Newly treated dummies were introduced twice a day into the experimental groups, exposing the workers to a daily amount of approximately 12 Qeq hydroxy acids. After three days the bees were killed and the in vitro rates of JH biosyntheses were measured as described above.

JH biosynthesis was expressed by two different indices: Index 1 was the mean of all the bees exposed to a given stimulus per experiment; Index 2, was the mean of only those bees that had the highest rate in each group of three exposed to a given stimulus per experiment. These latter bees probably represent the dominant bee established in each group (Larrere and Couillaud, 1993). Groups for which the data set was not complete were excluded from the analysis.

We also tested the effect of total body extracts on worker behavior. To minimize a priori bias by the observer, these experiments were performed blind. The experimental cages were marked by random numbers with no other indication for the observer of the type of treatment involved. On the second day of extract exposure, each group was observed for 20 min under dim red light. Agonistic interactions registered among workers included the number of threatening behavior and overt aggression events. Data were collected using a Psion organizer and analyzed using the Observer software (Noldus Information Technology b.v., Costerweg 5, P.O. Box 268, 6700 AG Wageningen, The Netherlands). Agonistic behavior was classified into two major categories: threatening behavior and overt aggressive behavior (Bloch et al., 1996). Briefly, threatening behavior included buzzing (fast short wing vibrations while opposite a nestmate), and pumping (distinct dorsoventral pumping movements with her abdomen) (Duchateau, 1989). Overt aggression included darting movements and attacks, biting or grappling with another bee, and pulling on a wing or hairs. (Doorn and Heringa, 1986; Duchateau, 1989; Bloch et al., 1996).

The different experiments conducted, the stimuli tested, the carrier and assay used, and the number of replicates for each experiment are summarized in Table 1.

Chemical Analyses and Quantification. Mandibular gland extracts, or their methylated products, were separated on a Tracor 540 GC using a 30-m SE 54 capillary column that was kept at an initial temperature of 60°C for 2 min and then heated to 250°C at 5°C/min, kept at 250°C for 5 min, and then raised to 295°C at 20°C/min. The presence of the hydroxy fatty acids was ascertained by coinjection with standard hydroxy acids or their corresponding methyl esters. Quantitative estimation was based on peak integration using 2.5 μg of eicosane as an internal standard.

Methylation of MG extracts was achieved with diazomethane prepared from 1 mmol (147 mg) 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) using an Aldrich Diazomethane Kit according to the manufacture's instructions.

TABLE 1. SUMMARY OF EXPERIMENTS CONDUCTED

Experiment	Stimulus tested	Carrier[a]	Assay	Replicates (*N*)	Results presented in
1	Total queen body extract	ODD, SWD	JH and behavior	8	Table 2
2	Queen exocrine gland extracts	ODD	JH	7	Table 3
3	Queen exocrine gland extracts	SWD	JH	9	Table 3
4	Queen MG extracts	Glass slide	JH	1	Table 4
5	Queen MG extracts	PD	JH	4	Table 4
6	Queen MG extracts	LCQ	JH	3	Table 5
7	Queen cuticular wash	LCQ	JH	4	Table 5
8	Hydroxy acids	ODD + wax	JH	5	Table 6

[a]ODD: oven-dried dummy; SWD: Soxhlet-washed dummy; PD: Petri dish covered with wax; LCQ: live callow queen.

RESULTS

Exposure of Callow Workers to Queen Total Body Extract (Experiment 1). In a preliminary experiment we examined the effect of exposing callow workers for one day to queen total body extracts using oven-dried dummies. The rates of JH biosynthesis in this experiment were similarly low for treatment (22.19 ± 1.8 SE, $N = 29$) and control groups (24.1 ± 2.7, $N = 25$; unpaired t test, $P = 0.55$). We therefore tested the effect of queen total body extracts over a longer period (three days). This is the period for which the differences in rates of JH biosynthesis between queenless and queenright workers are the highest (Röseler and Röseler, 1978; Bloch et al., 1996). In these experiments we used either oven-dried dummies or Soxhlet-washed dummies. Since there were no differences between these two modes of extract presentation (t test, index 1: $t = 1.62$, $df = 76$, $P = 0.11$; index 2: $t = 1.48$, $df = 24$, $P = 0.15$), the data were pooled. For the same reasons, the results of the experiments using chloroform–methanol and pentane–ethanol extracts were also pooled (t test, index 1: $t = 0.34$, $df = 43$, $P = 0.73$; index 2: $t = 0.13$, $df = 13$, $P = 0.15$).

Table 2 shows the effects of worker exposure to total queen extracts on the rates of JH biosynthesis in vitro and on worker behavior. Biosynthesis rates are expressed as the mean of all the workers tested for a given stimulus (index 1, see Methods and Materials) or the mean of only those individuals with the highest rates in each group, for all the groups tested to a given stimulus (index 2, see Methods and Materials). Irrespective of the index used, the rates in workers exposed to the extract were lower than in those exposed to solvent alone. In this respect, extracts made from queens collected from colonies before or after the

TABLE 2. RATES OF IN VITRO JH BIOSYNTHESIS AND FREQUENCIES OF THREATENING BEHAVIOR AND OVERT AGGRESSION IN 3-DAY-OLD WORKERS EXPOSED DAILY TO 1 QUEEN EQUIVALENTS OF TOTAL BODY EXTRACT OR TO SOLVENT[a]

	JH biosynthesis rate (pmol *l*shr/pair CA) mean ± SE (*N*)		Events per hour [mean ± SE (*N*)]	
Extract	Index 1	Index 2	Threatening behavior	Overt aggression
Solvent	47.3 ± 3.5 (27)*	62.8 ± 4.5 (9)*	11.4 ± 3.6 (15)NS	1.4 ± 0.7 (15)NS
Queen's total body extract	38.9 ± 2.4 (45)	51.3 ± 3.3 (15)	5.2 ± 1.6 (17)	1.2 ± 1.0 (17)

[a]Index 1: mean of all workers exposed to a given stimulus, index 2: mean for only those workers with the highest rates from each experimental group, for all groups exposed to a given stimulus. Statistical tests included unpaired *t* test for JH biosynthesis rate data and Mann-Whitney test for behavioral data. *Significance at a $P < 0.05$ level; NS: not significant.

onset of the competition phase were similarly effective (index 1: 36.9 ± 5.4 SE, $N = 15$; 39.6 ± 2.4 SE, $N = 34$ for queens before and during CPh respectively, *t* test, $df = 47$, $P = 0.6$; index 2: 53.8 ± 7.2 SE, $N = 5$; 50.4 ± 3.2, $N = 11$ for queens before and during the CPh respectively, $df = 14$, $P = 0.63$).

The behavioral observations following exposure to total queen extract revealed a more equivocal effect. Threatening behavior and overt aggression were slightly more frequent in the control groups than in the test groups, but these differences were not statistically significant (Table 2). The data presented in Table 2 comprise pooled data from different treatments and modes of exposure to extracts. Separate analyses demonstrated that there were no significant differences among workers from groups exposed to the different dummies (Mann-Whitney test, threatening behavior: $n = 36$, $P = 0.22$; overt aggression: $N = 36$, $P = 0.4$) or among experiments using the two extraction procedures (Mann-Whitney test, threatening behavior: $N = 36$, $P = 0.76$; overt aggression: $N = 36$, $P = 0.89$).

Effect of Queen Glandular Extracts (Experiments 2–7). The effects of various queen glandular extracts were tested using the above-described three-day bioassay with oven-dried dummies or soxhlet-washed dummies (Table 3). In experiment 2 using oven-dried dummies the rates of JH biosynthesis among workers exposed to queen glandular extracts were similar to those observed in bees that were exposed to solvent alone (unpaired *t* test, after Bonfferoni correction, $P > 0.01$). When index 1 was used the control group had, for unknown reasons, lower rates of JH biosyntheses (34.6 pmol/hr/pair) than control groups in the previous experiment (Table 2), and from those previously reported for queenless workers (Bloch et al., 1996). In contrast, when index 2 was used, the levels in the control group were as high as those measured in the previous experiment. Using index 2, JH production rates in workers exposed to mandibu-

TABLE 3. RATES OF IN VITRO JH BIOSYNTHESIS [MEAN ± SE (*N*)] BY CA OF 3-DAY-OLD QUEENLESS WORKERS EXPOSED DAILY TO QUEEN GLANDULAR EXTRACTS ON DIFFERENT DUMMIES

	Oven-dried dummies		Soxhlet washed dummies	
Extract	Index 1[a]	Index 2	Index 1	Index 2
Solvent	34.6 ± 5.3 (15)	59.2 ± 5.85 (5)	48.1 ± 3.9 (24)	62.7 ± 5.0 (8)
MG	33.0 ± 3.1 (24)	41.4 ± 4.9 (8)	49.1 ± 3.4 (17)	59.4 ± 4.7 (6)
HPG	38.7 ± 2.6 (24)	49.3 ± 3.6 (8)	41.5 ± 4.6 (18)	57.2 ± 6.25 (6)
SG	34.7 ± 4.6 (24)	52.5 ± 7.7 (8)	35.3 ± 5.0 (21)	58.2 ± 8.1 (7)
DG	38.9 ± 5.4 (15)	56.8 ± 10.2 (5)	36.5 ± 3.9 (21)	50.5 ± 4.9 (7)
TG	49.6 ± 6.3 (12)	63.2 ± 9.7 (4)	42.3 ± 4.3 (27)	60.8 ± 6.8 (9)

[a]Index 1 and index 2 are as explained in Table 2. MG: Mandibular glands, HPG: hypopharyngeal glands, SG: head salivary glands, DG: Dufour's gland, TG: tarsal glands. For description of the different types of dummies see Methods and Materials. There was no significant difference between treatments (ANOVA, $P > 0.05$).

lar glands extracts were reduced when compared to the control group (t test, $df = 11$, $P = 0.04$), but this difference became nonsignificant after Bonfferoni correction (P value has to be lower than 0.01).

In experiment 3, rates of JH biosynthesis for workers exposed to Soxhlet-washed dummies treated with solvent were higher than for workers exposed to oven-dried dummies treated with solvent (t test, index 1: $t = 2.1$, $df = 37$, $P = 0.047$; index 2: $t = 0.45$, $df = 11$, $P = 0.66$) and comparable to those in workers exposed to dummies treated with solvent in the total body extract experiment (experiment 1, Table 2). However, similar to experiment 2 with oven-dried dummies, we failed to detect any differences as a consequence of exposure to any of the queen glandular extracts (Table 3). There was a slight reduction in the rates of JH biosynthesis in workers exposed to SG or DG extracts (t test, $P = 0.049$, $P = 0.046$, respectively), but this proved to be insignificant after Bonfferoni correction.

The possible effect of the mandibular glands was specifically assayed by applying the extracts on glass slides (experiment 4) or onto small Petri dishes covered with honeybee wax (experiment 5). In these experiments too the rates of JH biosynthesis were similar for workers exposed to mandibular glands extracts or to solvent (Table 4).

In a separate set of experiments, live callow virgin queens were used as carriers for the stimuli. In experiment 6, mandibular gland extracts obtained from functional queens were applied to callow queens, and in experiment 7, cuticular washes of functional queens were applied to callow queens. Cuticular washes from functional queens resulted in significant inhibition of JH biosynthesis in the callow workers (Table 5, t test, index 1: $t = 2.74$, $df = 28$, $P =$

TABLE 4. RATES OF IN VITRO JH BIOSYNTHESIS [MEAN ± SE (*N*)] BY CA OF 3-DAY-OLD WORKERS EXPOSED DAILY TO 1 QUEEN EQUIVALENT OF MANDIBULAR GLAND (MG) EXTRACT OR SOLVENT[a]

	Solvent		Queen's MG extract	
Carrier	Index 1	Index 2	Index-1	Index-2
Glass slide	65.3 ± 9.9 (9)	89.3 ± 12.7 (3)	49.9 ± 11.5 (9)	89.7 ± 14.9 (3)
Petri dish	37.2 ± 3.3 (42)	56.7 ± 3.8 (12)	35.2 ± 3.2 (36)	57.2 ± 4.0 (11)

[a]Extracts were applied on glass slides (experiment 4) or on Petri dishes covered with wax (experiment 5). Index 1 and Index 2 are as explained in Table 2. There were no significant differences between treatments.

0.01; index 2: $t = 2.57$ $df = 8$, $P = 0.03$). Mandibular gland extract, on the other hand, had little effect. Similar rates of JH biosynthesis were observed in callows exposed to functional queen MG extracts and to clean solvent (Table 5). In this particular experiment, the rates of JH biosynthesis in the solvent control were unusually low. To test whether these low rates were due to inhibition of the CA or due to low performance of the specific incubation (e.g., bad methionine lot), we repeated the assay under the same incubation conditions and using the same methionine lot, but using callow workers (day 0) and 3-day-old queenless workers from the same colonies. These two groups represent minimal and maximal CA activities (Bloch et al., 1996). The results of these assays were 33.5 pmol/hr/pair ± 7.5 SE for 3-day-old workers and 10.8 ± 1.6 SE for callows. These are lower than those previously reported for similar age workers in our laboratory (49.8 ± 6.7 SE and 52.1 ± 5.4 SE for 3-day-old and 15.4 ± 3.6 SE for callows), but they still reflect the expected physiological differences between 3-day-old queenless and callow workers.

Effect of Mandibular Gland Hydroxy Acids (Experiment 8). The mandibu-

TABLE 5. RATES OF IN VITRO JH BIOSYNTHESIS [MEAN ± SE (*N*)] BY CA OF 3-DAY-OLD QUEENLESS WORKERS EXPOSED DAILY TO MANDIBULAR GLAND EXTRACT (EXPERIMENT 6) OR TO QUEEN CUTICULAR WASHES (EXPERIMENT 7)[a]

	Solvent		Treatment	
Queen extract	Index 1	Index 2	Index 1	Index 2
Mandibular glands	26.7 ± 3.5 (12)	38.4 ± 2.2 (4)	28.9 ± 3.6 (18)	41.2 ± 6.7 (6)
Cuticular wash	43.4 ± 4.6 (12)*	59.8 ± 6.1 (6)*	25.9 ± 4.2 (18)*	37.0 ± 5.9 (6)*

[a]Index 1 and index 2 are as explained in Table 2. Means were compared using unpaired t test; *$P < 0.05$.

TABLE 6. RATES OF IN VITRO JH BIOSYNTHESIS [MEAN ± SE (*N*)] BY CA OF 3-DAY-OLD QUEENLESS WORKERS EXPOSED DAILY TO HYDROXY ACID BLEND OR SOLVENT APPLIED ON OVEN-DRIED DUMMY AND WAX BALL[a]

Extract	Index 1	Index 2
Solvent	40.1 ± 3.1 (36)	51.0 ± 5.8 (12)
Hydroxy acids blend	33.2 ± 2.7 (47)	48.7 ± 4.1 (16)

[a]Index 1 and index 2 are as explained in Table 2. Using both indices, differences between hydroxy acid blends and the control group were not significant (unpaired t test, $P > 0.05$).

lar glands of queen *B. terrestris* are rich in 3-hydroxy fatty acids. We therefore tested in our bioassay whether they possess an inhibitory activity. We first estimated their amount in functional queens by GC analysis of the methylated extracts. Queen glands possessed an average of 1.53 μg ± 0.39 of 3-HOA, 2.38 μg ± 0.68 of 3-HDA and 0.23 μg ± 0.08 of 3-HDDA (mean ± SE for six queens). Accordingly, callow workers were exposed daily to about 12 queen equivalents of synthetic hydroxy acids. There was a slight, but not significant decrease in the rates of JH biosynthesis in workers exposed to the hydroxy acid blend (two-way ANOVA, treatment: $df = 1$, $P = 0.09$; Table 6).

DISCUSSION

Primer pheromones regulating reproduction are predicted to affect the physiological processes involved in ovarian development. In *B. terrestris* workers, reproduction is effectively inhibited until a particular period in colony development. It was postulated that the queen actively inhibits the workers from reproducing by combined behavioral dominance and pheromone production (Honk et al., 1980; Röseler et al., 1981). The onset of worker reproduction was accordingly assumed to be the result of a decline in pheromonal output by the queen (Pomeroy and Plowright, 1982; Doorn, 1987; Duchateau, 1989; Doorn and Chrambach, 1989). Based on surgical removal and in vitro assays, the mandibular glands have been pinpointed as the source of the putative pheromone, but the chemistry of its secretion has not been elucidated. However, a recent finding that queens before the competition phase (a period when worker reproduction is effectively inhibited) and queens after the competition phase (when worker reproduction takes place) can equally inhibit worker reproduction in small groups (Bloch et al., 1996) casts doubt on these conclusions. In an effort to identify the putative mandibular gland pheromones, a comprehensive study of the chemistry of the secretion in mated queens was performed (Hefetz et al., 1996). In this article we reevaluate the occurrence of a primer pheromone that is responsible for the inhibition of worker reproduction. Because JH is apparently a gonadotropin

in *B. terrestris* (Röseler and Honk, 1990; Bloch et al., in preparation), we used rates of JH biosynthesis to assess the inhibitory activity of queen secretions.

Rates of JH biosynthesis were significantly inhibited in queenless workers exposed daily to total body extracts (experiment 1, Table 2) or cuticular washes (experiment 7, Table 5) of a functional queen. This suggests that the extract contains substances that may act as primer pheromones and that these are apparently spread on the surface of the queen's body. The same extracts, however, did not significantly alter the aggressive behavior that usually erupts among workers under queenless conditions, at least until dominance hierarchy is achieved. Threatening behavior and overt aggression in groups of workers exposed to queen total body extracts were low, but did not differ from the control groups (experiment 1, Table 2). It is thus possible that the putative pheromone affects the physiology, but not the behavior of the workers. This lends credence to earlier suggestions that in *B. terrestris* the queen uses mixed behavioral and pheromonal means to inhibit worker reproduction (Honk et al., 1980; Röseler et al., 1981).

To identify the glandular source of the prospective pheromone, we repeated the assay used to compare the effect of total body extracts, but this time we exposed callow bees to dummies treated with extracts of five major queen exocrine glands (experiments 2 and 3, Table 3). When we used oven-dried dummies, the low rates of JH biosynthesis in the control were also significantly lower than those obtained previously. The rates of JH biosynthesis in workers exposed to the glandular extracts were similarly low, but because of the low control rates we could not conclusively state that the glands are devoid of activity. The effect of the nontreated oven-dried dummies could have been due to odors emitted by the dummy that affected the workers irrespective of the treatment. In experiment 3, therefore, we tried to eliminate all possible odor from our dummy queens by extensively washing them in a Soxhlet apparatus. This procedure resulted in higher levels of JH biosynthesis in the control. Nevertheless, none of the examined gland extracts exerted significant inhibition on worker CA activity (Table 3). Thus, irrespective of the dummies used, none of the gland extracts possessed primer pheromone activity.

These results are not consistent with previous reports that the mandibular glands are the source of the primer pheromone that inhibits worker reproduction in *B. terrestris* (Honk et al., 1980; Röseler et al., 1981). We therefore further examined the effect of queen MG extracts in supplementary experiments, using different application methods. The results confirmed that the mandibular gland secretion has no effect on in vitro biosynthesis of JH. There were no significant differences between callows exposed to queen MG extracts or to solvent applied on glass slides (experiment 4), a Petri dish covered with wax (experiment 5), or a live callow gyne body (experiment 6). In the experiments using virgin queens as carriers for the tested material, the control was rather low. Thus, although

the levels of JH were not significantly different from the control, the results are equivocal. In order to validate these results we measured the rates of JH biosynthesis for 3-day-old queenless workers (expected to have high JH synthesis) and for callow workers (expected to have low JH synthesis) under the same incubation conditions and the same lot of radioactive methionine. The synthesis rates for JH in both the 3-day-old queenless workers and in callows were indeed lower than observed in earlier studies, and significantly lower in the callows than in the 3-day-old queenless workers. This indicates that the results obtained by using virgin queens as carriers are valid. Moreover, when we used this system in the application of cuticular washes, we obtained significant inhibition of JH biosynthesis. We also tested the activity of three major hydroxy acids present in the mandibular glands of queen *B. terrestris* (Hefetz et al., 1996). Application of 12 Qeq/day did not change the rates of JH biosynthesis when compared to the control.

The differences between the results obtained in the present study and those of previous studies (which suggested that the mandibular glands are the main source for *B. terrestris* queen pheromone) could be due to differences in performing the bioassays: (1) Röseler et al. (1981) measured CA volume as opposed to our direct measurement in the in vitro assay. We consider direct measurement of JH biosynthesis to be more reliable. Studies with a wide variety of species, including the related honeybee, demonstrated a poor correlation between these indices (e.g., Lanzrein et al., 1978; Feyereisen, 1985; Dale and Tobe, 1986; Huang et al., 1991; Kaatz et al., 1992). (2) The workers assayed in Röseler et al. (1981) were 1 day old vs. 3 days old in the present study. We selected this age because the CA of 3-day-old, but not that of 1-day-old, workers shows a maximum difference in activity between queenless and queenright callow workers (Röseler and Röseler, 1978; Bloch et al., 1996). (3) In the present study the queen dummies and workers were maintained without any brood, whereas Röseler et al. (1981) added 10–20 pupae to each experimental group. Although we do not know the precise role of brood in social interactions in bumblebees, a brood pheromone that inhibits ovarian development in workers was described for honeybees (Arnold et al., 1994). (4) The dummies in our study were virgin queens, while Röseler et al. (1981) used bodies of functional queens that had not caused inhibition in previous assays. (5) Röseler et al. (1981) mixed wax obtained from normally developing colonies with their extract preparation, while in this study only the solvent was used. It is possible that the supplementary components that originate from normally developing colonies in the assays performed by Röseler et al. (1981) affected their results.

The question of whether queen *B. terrestris* possess a primer pheromone that inhibits worker reproduction is still unresolved. Supporting the presence of such a pheromone are the inhibitory activities of the total body extract or of cuticular washes. We have not been able to determine whether the pheromone

is produced by an internal exocrine gland and is applied onto the epicuticle of the bees by self-grooming or whether it is an integral part of the cuticular lipids. According to our results, none of the commonly known exocrine glands, mandibular gland included, are active. It is possible that other glands, not studied by us, produce the substances, that the activity of the pheromone comprises combined secretions from several glands, or that a better assay is needed for exploring the existence of a primer pheromone that inhibits worker reproduction in bumblebees.

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BIOCHEMICAL FUNCTIONS OF GEOPHAGY IN PARROTS: DETOXIFICATION OF DIETARY TOXINS AND CYTOPROTECTIVE EFFECTS

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Abstract—This study tests hypotheses on the biochemical functions of geophagy in parrots: mechanical enhancement of digestion, acid buffering capacity, mineral supplementation, adsorption of dietary toxins, and gastrointestinal cytoprotection. Parrots showed clear preferences for specific soil horizons. Comparisons of preferred and nonpreferred soils from several sites suggest that soils have little ability to enhance grinding and no measurable ability to buffer gastric pH. Soils offered insignificant mineral supplementation since most minerals occurred at similar levels in samples regardless of preference, and the minerals were generally more plentiful in the birds' diets. Sodium was available in moderate levels at some sites (>1000 ppm), but was well below sodium detection thresholds of parrots. X-ray diffraction, cation exchange capacity, and in vitro adsorptive trials showed that the preferred soils are capable of exchanging substantial quantities of cations and are capable of adsorbing low-molecular-weight secondary compounds. In captive *Amazona* parrots, orally administered clay reduced the bioavailability of the alkaloid quinidine by roughly 60%, demonstrating that in vivo adsorption of potentially

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toxic compounds may be a biologically important function of geophagy. Labeled clay remained in the lower gastrointestinal tract of captive parrots for >12 hr, which along with high adsorptive capacities, further suggests a potential role in protecting the gastrointestinal lining from various biological and chemical insults. Detoxification and cytoprotection are the most likely functions of geophagy for parrots and herbivores with similar ecologies. Given the variety of chemically defended seeds consumed by these herbivores, geophagy likely protects consumers from dietary toxins, allowing increased diet breadth and/or enhancing digestibility.

Key Words—Parrot, macaw, geophagy, clay, mineral, detoxification, secondary compounds, dietary ecology, cytoprotection.

INTRODUCTION

The consumption of soil (geophagy) is widespread among vertebrates, particularly herbivores, and is perhaps best known in ungulates' visiting salt-licks (Jones and Hanson 1985). Geophagy is also common in a variety of nonhuman primates (Izawa, 1993; Mahaney et al., 1996), indigenous and modern human cultures (Laufer, 1930; Cooper, 1957; Hunter, 1973; Johns, 1986; Abrahams and Parsons, 1996). Soil-eating has also been documented in koalas (Smith, 1979), reptiles (Sokol, 1971; Marlow and Tollestrup, 1982), and in many seed-eating birds (Pendergast and Boag, 1970; Munn, 1992; Pryce, 1994; Gionfriddo and Best, 1995). Exactly what drives animals and humans to eat soil remains unclear, but the answers might yield insight into the interactions of plant chemistry and vertebrate physiology, as well as shedding light on the origins of self-medication in humans and other vertebrates.

Although one clay lick used by birds and mammals in Peru has been known for some time (Emmons and Stark, 1979; Terborgh, 1983), recent work has found that geophagy is not only geographically widespread in South America, but also occurs in a variety of herbivores and omnivores (C. Munn unpublished data). Reports of parrot geophagy in the Neotropics have now been reported from Mexico, Peru, Bolivia, and Brazil, and the behavior has been observed in many parrots, cracids, pigeons and doves, monkeys, tapirs, and peccaries (Heyman and Hartman, 1991; Munn et al., in preparation; E. Enkerlin, personal communication, P. Roth, personal communication). Similar aggregations of parrots and other herbivorous birds at clay licks have recently been described in Irian Jaya (Diamond et al., 1999), and Grey Louries in Botswanna (*Corythaixoides concolor*) (Pryce, 1994). As this behavior is often overlooked, it is expected that new observations will continue to be made and support the notion that avian geophagy is widespread and has evolved several times independently.

Many hypotheses have been proposed to explain geophagy, the best known being: (1) mechanical enhancement of digestive grinding by large particles (grit)

(cf. Best and Gionfriddo, 1991); (2) supplementation of nutrition by release of minerals (Jones and Hanson, 1985; Kreulen, 1985; Hunter, 1993); (3) buffering gastrointestinal pH (Oates, 1978); (4) reduction of toxicity of food by adsorption of plant toxins to clay (Hladik and Gueguen, 1974; Johns, 1990; Johns and Duquette, 1991); and (5) enhancement of the ability of the gastrointestinal tract to protect itself from chemical insult (cytoprotection) by induction and alteration of mucous secretion (Rateau et al., 1982; Kreulen, 1985; Vermeer and Ferrell, 1985; Mahaney et al., 1996). Thus, there are several possible, nonexclusive functions of geophagy in vertebrates.

Given the number of known clay-licks in Peru, and our growing knowledge of the ecology of the birds visiting these sites (Emmons and Stark, 1979; Gilardi and Munn, 1998), this area presented a unique opportunity to conduct an in-depth study of the phenomenon of geophagy, and to test directly these hypotheses on the functions of soil-eating in parrots. We conclude that mechanical enhancement of digestion, buffering of the gastrointestinal tract, and mineral supplementation are unlikely to be the primary cause of geophagy. Rather, geophagy may provide substantial alimentary detoxification of charged secondary compounds and may protect the gut lining from various chemical challenges. For herbivores that consume chemically well-defended foods, soil-eating would thus allow increased diet breadth and/or enhance digestibility of these otherwise toxic resources.

METHODS AND MATERIALS

Sample Collection. We collected soil samples from clay-licks in Manu National Park (11°57′S; 71°17′W) in August 1992 and 1993 and in the Tambopata–Candamo Reserved Zone (13°10′S; 69°30′W) in February 1994. At each site, birds and/or mammals show a clear preference for particular soils, which we noted either by direct observation of the animals or the bill/dental impressions left in the soil. In Manu, this horizon ran for hundreds of meters along the river bank, whereas in Tambopata, the exposed cliff is much higher (≈30 m) and the soil horizons less well-defined. At this site, birds consume soil from some patches on the cliff face more than others. At all sites, we collected samples from both the preferred and nonpreferred areas; weighed, dried, and gently ground the samples to a course powder; and stored them for all subsequent analyses.

Particle Size and Cation Exchange. We determined clay, silt, and sand fractions of the soil samples using standard methods (sand 2 mm–50 μm, silt 50–2 μm, and clay <0.2 μm) (Gee and Bauder, 1986). Briefly, we dispersed the samples in dilute aqueous sodium hexametaphosphate, passed them through a sieve to remove the sand, resuspended the clay and silt, and sampled the suspension at a fixed time and depth to determine clay content. We dried these fractions

and determined silt content by subtraction. We determined the cation exchange capacity for clays from all licks using a barium method in the DANR laboratory at UC Davis (Janitsky, 1986) and present the results in units of milliequivalents per 100 g.

Mineral Release. We analyzed all samples for mineral release using two extracts, one approximates the extractive conditions of a bird's digestive fluid and a second is exhaustively extractive and provides an estimate of total mineral content. These are referred to hereafter as available and total mineral content, respectively. To estimate available minerals, we added 1 g powdered soil to 15 ml aqueous HCl at pH 2.0, agitated the mixture at 38°C for 1 hr, and then centrifuged it (cf. Hunter and De Kleine, 1984). To estimate total minerals, we added 1 g of powdered soil to 0.5 ml concentrated HNO_3 and 2 ml 30% H_2O_2 in a Teflon vessel and heated it under pressure in a microwave oven (5 min at 40% power, 8 min at 90% power) (CEM Corp. MDS 2000). We diluted these extracts to 15 ml with H_2O, left them to settle, and later analyzed an aliquot of all extracts using inductively coupled plasma optical emission spectroscopy (Thermo Jarrell Ash Atomscan 25) for the following elements: Li, Na, Mg, P, S, K, Ca, Cr, Mn, Fe, Co, Cu, Zn, Se, Mo (for use of ICP on soils, see Wang et al., 1989). We converted raw results to milligrams per kilogram of dry weight using three-point standard curves based on reference solutions for the appropriate element (Fisher Scientific).

From preliminary analyses, we found that minor changes in the ratio of extract solution to soil had a marked effect on the quantity of minerals released on a per gram of soil basis. To determine this relationship, we analyzed a series of samples varying the extract–soil ratio, tested for eight elements (Na, K, Mg, Ca, Fe, S, P, Zn), and present the results in terms of the quantity of mineral released per gram soil as a function of extract–soil.

For particle size, cation exchange capacity (CEC), and mineral content, we compared mean values of preferred soils with those of nonpreferred samples. In the few cases where the preferred values were either higher or lower than nonpreferred values, we compared means using a planned comparison analysis of variance.

Buffering Capacity. We determined the buffering capacity of soils by adding incremental amounts of HCl to soil or antacids in aqueous suspension under constant agitation (Watts, 1994). We added 7-g samples of soil or prescribed doses of over-the-counter antacids to 30 ml distilled H_2O and agitated this $\geq$10 min. We then added sequentially 2-ml aliquots of HCl (1 M) and recorded the pH after stabilization.

Clay Mineralogy. We analyzed soils from three sites in Manu and the main Tambopata lick for clay mineralogy using standard X-ray diffraction methods (Whittig and Allardice, 1986). Briefly, a small sample of the clay fraction is placed on a pair of quartz plates and each is saturated with different salts. The

crystalline structures of different clay types react differently to a series of chemical and/or heat treatments. We present the results for each plate with the subsequent analyses to aid in identification of or loss of peaks that are diagnostic of specific clay minerals. Although we analyzed several samples from each site, we present data only from Manu since the mineralogy of all Peruvian samples was similar.

In Vitro Adsorption. We determined the adsorption isotherms of soils for two compounds, the polyphenolic gallotannin (Fluka) and the alkaloid quinine (Aldrich), in order to estimate the affinity of these chemicals for soils in a simulated gastric environment. We suspended 1 g of soil in 100 ml of a solution mimicking avian gastric fluid (0.1 N NaCl, pH 2.0 HCl) (Hunter and De Kleine, 1984) and drew off 5-ml aliquots during agitation, transferring them to 15-ml centrifuge tubes. We added varied concentrations of these model compounds to these seven tubes, agitated the samples for 30 min at 38°C, and then centrifuged them at 16,000*g* for 2 min. We assayed aliquots of the parent compound on a spectrophotometer (Schimadzu) by comparison with standard colorimetric curves using gallotannin (270 nm) and quinine (346.3 nm). Adsorption on clays via cation exchange can be modeled with a Langmuir adsorption isotherm, which provides results in the form of an adsorptive maximum and estimates the quantity of compound a clay will adsorb when saturated (Heimenz, 1986).

Brine Shrimp Bioassay. To test whether soils can reduce the toxicity of parrot foods, we extracted seven of the most toxic seeds in macaw diets (Gilardi and Munn, in preparation) and exposed the extracts to a realistic quantity of soil in a simulated gastric environment. We then compared the toxicity of soil-exposed extracts to controls using a brine shrimp bioassay (Solis et al., 1993). We extracted 1 g of plant material in 20 ml 100% methanol for 24 hr, split these extracts into two portions, and dried them in vacuo. To each dried extract and to two standards (quinidine and digitonin), we then added 10 ml simulated gastric fluid to each (pH 2 HCl, 0.1 N NaCl), and added 250 mg dry soil to one and nothing to the other (control). After agitating them at ≈40°C for 30 min, we titrated them to pH ≈7 with 0.1 M NaOH, and centrifuged at them at 4000*g* for 10 min. Using a 12-well tissue culture plate (Falcon), we added 0, 20, and 200 μl and 1 ml of extract in triplicate, and artificial seawater (InstaOcean) to make 5 ml total. We then added 10–30 brine shrimp nauplii to each well and counted live and dead shrimp at 24 h and determined the LD_{50} using probit analysis (Polo Software).

In Vivo Detoxification. To test detoxification in vivo, we dosed eight orange-winged Amazons (*Amazona amazonica*) with weight-specific quantities of the alkaloid quinidine sulfate (150 mg/kg, Aldrich). Although it would have been preferable to use quinine because of its biological relevance and because we used that compound in the in vitro work, we selected the stereoisomer of quinine, quinidine, as our model alkaloid because it is far less toxic to verte-

brates and the available assay methods are more sensitive. For our purposes in testing the in vivo detoxification mechanism, we assume that absorption by the parrot and adsorbtion by the clay of this model compound were sufficiently representative of a low-molecular-weight alkaloid to be of value. We simultaneously treated half the birds with 4 ml H_2O, and half with 4 ml clay suspension using preferred Peruvian soils (≈0.5 g/ml), reversing the water and clay treatments on the same individual birds 14 days later. We administered the clay/water/drug with a stainless-steel feeding tube modified with a short section of plastic tubing on the tip, which held a gel capsule containing the quinidine (size 5 capsules, Chemical and Pharmaceutical Industry, Ramsey, New Jersey). We sampled blood (0.5 cc/draw) at 1, 2, and 3 hr after dosing from either the jugular or the median ulnar vein using 1-cc tuberculin syringes, and stored them in heparinized blood collection tubes (Microtainer, Becton Dickinson & Co., Franklin Lakes, New Jersey). We centrifuged these samples at 150*g* for 5 min to pellet the red blood cells, and assayed the plasma using a TDx fluorescence polarization immunoassay with reagents for quinidine (Abbott Inc., Abbott Park, Illinois) (Ronald, 1984). We determined circulating drug levels by comparison with a nonlinear standard curve determined from quinidine in *A. amazonica* blood. We analyzed treatment differences using repeated measures analysis of variance (BMDP 2V) (Dixon, 1992).

Soil Passage Rate. To estimate the distribution and transit time of a dose of clay in the gastrointestinal tract of captive parrots, we gavaged a blue-fronted Amazon (*Amazona aestiva*) with 6–8 cc of iodinated contrast fluid (Isovue-200, Bracco Diagnostics, Princeton, New Jersey), and another with iodine-saturated clay (6–8 cc). We fasted both birds for 1 hr before initial dosing and returned them to ad libitum food 4 hr later. Following administration of clay or contrast agent, we took lateral abdominal radiographs at 30 min and at 1.5, 4, 8, 12, and 24 hr.

RESULTS

Particle Size and Cation Exchange. All soil samples contained substantial quantities of clay; preferred soils contained significantly more than nonpreferred sites (50% vs. 35% respectively; Table 1). The elevated clay levels in the preferred sites translates to decreased sand content in these samples, and silt levels were nearly equivalent among the three sample types. These high clay contents correlated well with the relatively high cation exchange capacities (CECs) ($R^2 = 0.235$, $P = 0.047$; Figure 1), and preferred soils had significantly higher CECs than non-preferred samples ($P = 0.019$; Table 1).

Mineral Release. There were marked differences between the two extracts used, the total extract yielding higher values for a given mineral than the avail-

TABLE 1. PARTICLE SIZE (%), CATION EXCHANGE CAPACITY (meq/100 mg), AND AVAILABLE MINERALS (mg/kg) AT PERUVIAN GEOPHAGY SITES[a]

Site	Sample[b]	Clay	Silt	Sand	CEC	Ca	Co	Cr	Cu	Fe	K	Li	Mg	Mn	Mo	Na	P	S	Se	Zn
Near	above	13.3	20.8	65.9	11.7	318	bdl	0.35	0.09	4.85	13.7	0.12	376	6.26	0.02	469	0.05	0.88	5.25	1.37
	prefer	47.9	51.3	0.78	30.5	280	0.07	0.21	0.20	3.19	13.6	0.33	388	3.06	bdl	1565	0.48	0.97	bdl	0.71
	below	40.6	52.5	6.84	30.5	408	0.00	0.32	0.06	4.02	11.1	0.35	491	10.7	bdl	1499	0.20	0.93	bdl	2.84
Main	above	52.6	43.4	4.06	20.0	757	nd	nd	nd	6.12	14.1	nd	592	nd	nd	415	0.10	0.43	nd	2.49
	prefer	55.6	42.1	2.32	33.0	309	0.07	0.40	0.04	4.15	9.07	0.39	452	8.78	bdl	1749	0.07	0.08	bdl	1.11
	below	27.8	43.3	29.0	20.5	303	0.15	0.35	0.06	8.99	28.8	0.34	499	11.6	bdl	1598	0.72	12.14	bdl	3.40
Arma	above	25.1	46.7	28.2	17.0	341	0.48	0.21	0.29	6.89	22.6	0.16	387	15.8	bdl	457	6.89	6.72	bdl	2.94
	prefer	44.5	41.0	14.5	27.5	379	0.17	0.25	0.26	4.66	23.7	0.32	381	10.2	bdl	969	0.22	0.73	bdl	2.49
	below	40.3	47.6	12.1	18.5	275	0.36	0.19	0.24	4.33	28.5	0.26	284	15.2	bdl	685	0.15	0.20	bdl	4.01
Tamb[c]	above	37.5	59.3	3.20	15.3	881	1.33	0.27	0.64	113.4	271.9	0.34	726	86.6	0.00	117	104.0	3.43	0.00	21.2
	prefer	81.7	17.3	1.02	20.8	610	0.44	0.06	0.82	73.1	356.4	0.47	824	122	0.00	380	0.45	0.56	0.00	3.12
	below	38.0	48.9	13.0	13.5	317	0.55	0.04	0.56	111.1	196.3	0.18	465	77.4	0.00	75.4	0.41	0.32	1.35	5.24
Mean	above	32.1	42.5	25.3	16.0	574	0.60	0.28	0.34	32.8	80.6	0.20	520	36.2	0.01	365	27.8	2.87	1.75	6.99
	prefer	57.4	37.9	4.64	28.0	395	0.19	0.23	0.33	21.3	100.7	0.38	511	35.9	0.00	1166	0.30	0.58	0.00	1.86
	below	36.7	48.1	15.2	20.8	326	0.26	0.22	0.23	32.1	66.2	0.28	435	28.8	0.00	964	0.37	3.40	0.34	3.87
P =		0.026		0.195	0.019						0.745	0.030				0.176				
Minerals from plant resources (see text)						4738	bdl2	bdl2	8.43	62.42	16784	bdl2	2911	62.13	bdl2	36.71	3611	2088	bdl2	27.8

[a]Near: a small lick =100m downstream of the "main" lick along the Manu River. Main: the central lick along the Manu river 71°25′30″ W, 11°50′ S; =1 hr upstream of Cocha Cashu Biological Station. Arma: a small lick on a tributary off the Manu that is visited primarily by *Ara macao*. Tamb: ; nd: no data; bdl: below detectable levels; bdl2: most samples below detectable levels.

[b]Collected 1 m above, below, or at the preferred soil horizon.

[c]Means of three subsamples.

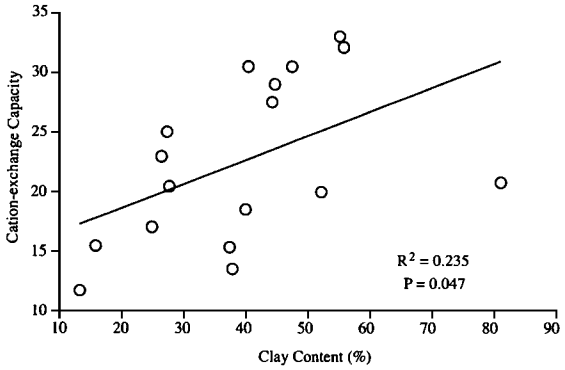

FIG. 1. The relationship of clay content with cation-exchange capacity in soils from geophagy sites in southeastern Peru.

able extract (Table 2). Viewing this difference as extraction efficiency, three elements were relatively available (sodium, calcium, and sulfur), while the others were <15% available. The large difference in iron (<1% available) suggests that the microwave/acid process degrades the crystalline lattice of the clay, releasing large amounts of iron. Within the available extracts, the ratio of clay to water had a profound effect on the minerals released (Figure 2). As the amount of clay was increased in a given volume of water, the amount of minerals released on a per-gram-of-soil basis decreased sharply.

All soils released substantial quantities of some minerals and virtually none of some others (Table 1). Only potassium, lithium, and sodium were released in greater quantities in comparing preferred vs. nonpreferred samples, and only the content of Li was significantly different.

Buffering Capacity. Preferred soils from Manu and Tambopata showed virtually no ability to buffer pH; pH values for the clay suspensions stabilized between 3 and 6 orders of magnitude lower than over-the-counter antacids and were indistinguishable from distilled water (Figure 3). For instance, the soils stabilized at pH 1–1.5 and the antacids between pH 4 and 6.5.

Clay Mineralogy. X-ray analysis of the clays shows that all the Peruvian clays contained smectites, kaolin, and mica (Figure 4). The Manu samples were predominantly smectite, and the Tambopata samples showed greater proportions of kaolin and mica. The smectites are well known for having high surface areas and high CECs, whereas mica and kaolin are intermediate in these properties. Thus, the cation exchange results concur with the clay mineralogy findings in that the Manu clays were both high in smectites and had similarly high CECs. In contrast, the Tambopata samples had little smectite, and despite the higher clay content, had lower CECs.

TABLE 2. DIFFERENCES IN MINERALS RELEASED BY PERUVIAN SOILS EXTRACTED BY MICROWAVE/ACID (MA) VS. SIMULATED GASTRIC CONDITIONS (GC)

Sample and extract	Parts per million							
	Na	K	Ca	S	P	Mg	Fe[a]	Zn
MC05								
GC	1,320	13.9	714	0.550	0.048	589	6.82	0.681
MA	1,280	954	1,850	0.806	284	7,310	21,500	91.2
% Δ	−2.96	98.5	61.4	31.8	100	91.9	100.0	99.3
MC08								
GC	969	23.7	379	0.727	0.217	381	4.66	2.49
MA	881	722	779	1.38	384	3,090	21,500	69.1
% Δ	−10.0	96.7	51.3	47.2	99.9	87.7	100.0	96.4
MC10								
GC	1,570	13.6	280	0.97	0.484	388	3.19	0.711
MA	149	949	934	5.75	152	4,630	21,600	64.7
% Δ	−4.89	98.6	70.0	83.2	99.7	91.6	100.0	98.9
T1A								
GC	123	299	850	2.75	85.6	736	115.3	19.0
MA	174	1,371	1,210	4.27	468	3,990	21,500	145
% Δ	29.3	78.2	30.0	35.6	81.7	81.6	99.5	86.9

TABLE 2. CONTINUED

Sample and extract	Parts per million							
	Na	K	Ca	S	P	Mg	Fe[a]	Zn
T4P								
GC	332	345	573	0.642	0.595	812	32.9	4.32
MA	360	2,010	1,260	0.643	154	4,690	21,500	95.4
% Δ	7.60	82.8	54.5	0.249	99.6	82.7	99.8	95.5
T8B								
GC	63.0	183	279	0.323	0.595	407	94.0	4.99
MA	88.2	1,110	255	1.06	311	1,930	21,500	68.6
% Δ	28.6	83.6	−9.69	69.6	99.8	78.9	99.6	92.7
Chaco								
GC	647	374	2,195	136	382	749	189.7	11.6
MA	964	2,920	6,460	368	686	9,570	12,600	130
% Δ	32.9	87.2	66.0	63.0	44.2	92.2	98.5	91.1
Extraction efficiency (%)	88.5	10.6	53.8	52.8	10.7	13.4	0.386	5.61

[a] Microwave/acid extraction for Fe was clearly saturated at this dilution; these figures should therefore be interpreted as minimum values. M samples from Manu, T samples from Tambopata, and Chaco is a soil sold on the streets in Cusco, Peru for gastric irritation, included here for comparison.

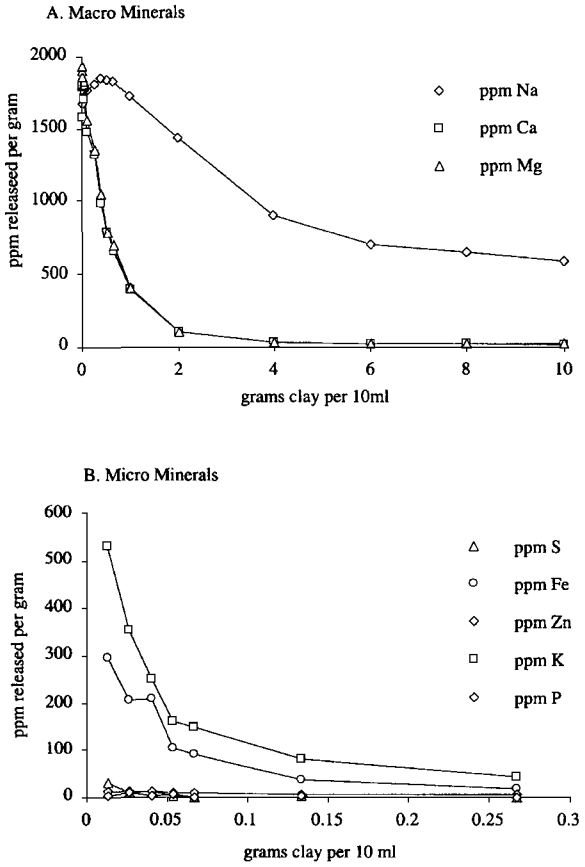

FIG. 2. Minerals released from soils as a function of concentration in solution.

In Vitro Adsorption. These generally high CECs for preferred soils (≈28 meq/100 ml) were correlated with the soils' abilities to adsorb secondary compounds. For both classes of organic compounds tested in vitro, preferred soils from both Tambopata and Manu showed adsorptive maxima greater than pure kaolin and less than pure bentonite (Figure 5). The adsorptive maxima figures suggest that the preferred soils can adsorb up to 90–125 mg quinine and 6–8 mg tannic acid per gram of ingested soil in simulated gastric conditions.

Brine Shrimp Bioassay. For the plant foods tested, exposure to soil in a simulated gastric environment caused a greater than threefold reduction in toxicity as measured by LD_{50} in brine shrimp (Figure 6). Soil caused a twofold reduction in the toxicity of the quinidine but had no measurable effect on the

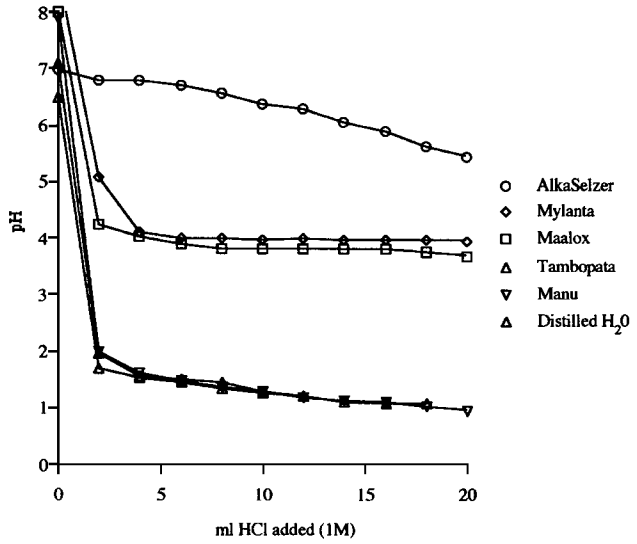

FIG. 3. Buffering capacities of Peruvian soils compared with over-the-counter antacids.

saponin digitonin. Given that the alkaloid is positively charged at pH 2, and the saponin is not, the detoxification is likely occurring via cation exchange.

In Vivo Detoxification. Circulating quinidine levels in birds dosed with clay were reduced by ≈60% compared to birds dosed with water during the first 3 hr after ingestion ($P < 0.001$, Figure 7). Peak levels of the drug were reduced from ≈1.5 to ≈0.6 μg/ml. Parrots required a very high drug dose to attain detectable levels; 150 mg/kg quidine produced circulating levels of ≈1.5 μg/ml. In contrast, a 3–4 mg/kg dose in humans produces levels of 2–5 μg/ml (Goodman et al., 1990).

Soil Passage Rate. Clay was present in the crop, proventriculus, and gizzard at 30 min after administration and was clearly present in the lower gastrointestinal tract 12 h later (Figure 8a). No clay was visible at 24 h. Pure contrast agent behaved in much the same way, but most was cleared more rapidly, being present only in the cloacal area at 8.5 hr (Figure 8b). Two caveats are worth mentioning. Since birds and mammals eat dry clay, a crop full of lumps of clay is likely to take hours to hydrate and be passed from the muscular stomach to the lower gastrointestinal tract. Moreover, from preliminary testing, the iodine-saturated clay was much less radiodense than pure contrast agent; thus, only large quantities were visible. More natural dosing, more effective labeling, or more sensitive detection would all likely reveal still longer-term presence of clay in the gastrointestinal tract.

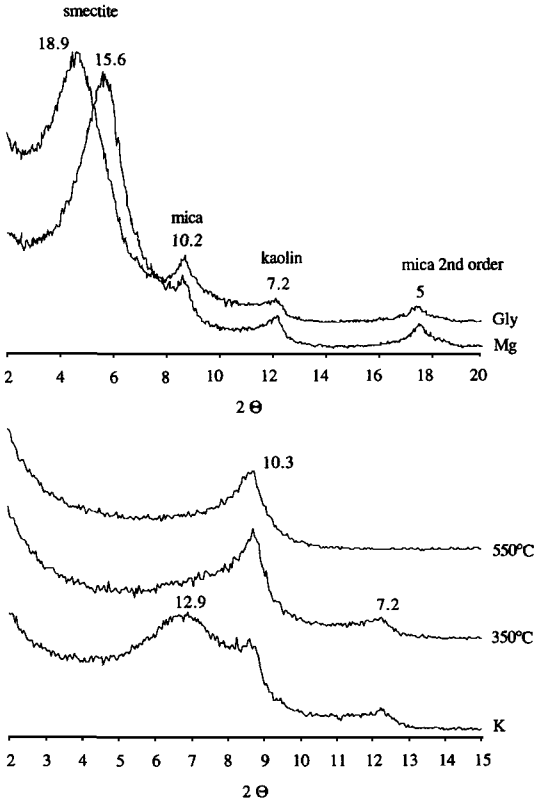

FIG. 4. Clay mineralogy of Manu soil. The top two curves illustrate the spectrum of the sample saturated with magnesium and subsequently with glycerol. The bottom three curves show the potassium saturated curve and subsequent heat treatments of that sample. Peaks occur at differing incident angles (2 theta) of the radiation source and correspond to the abundance of various clay minerals with different crystalline structure (indicated by D-space values (Å) above each peak. Under different chemical or thermal treatments, the crystalline structures of the different clay families change and facilitate the identification of the minerals present.

DISCUSSION

Geophagy is a multifaceted phenomenon, as evidenced by the diversity of animals that exhibit this behavior in a variety of ecological settings. In this investigation we address the question of geophagy in parrots and test several hypotheses on the causes of soil-eating in vertebrates: grit for mechanical enhancement of digestion, mineral supplementation of a deficient diet, acid buffering, adsorp-

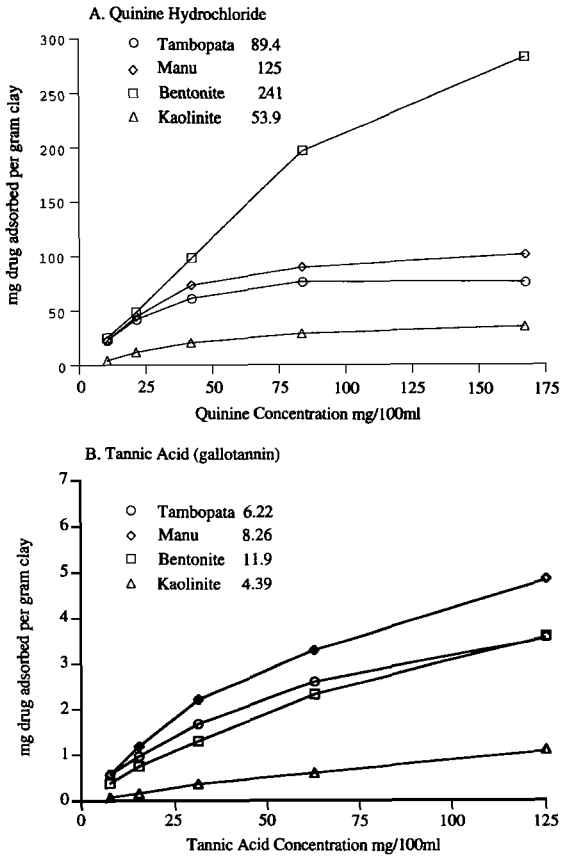

FIG. 5. Langmuir adsorption isotherms of quinine and tannic acid on Peruvian soils and clay standards in a simulated gastric environment; legends show sample identity and the adsorptive maximum (mg/g).

tion of dietary toxins, and gastrointestinal cytoprotection from chemical insults. Our analysis of soils from clay licks in Peru and experiments with captive parrots permitted us to address the validity of the above hypotheses; a brief discussion of each follows.

Mechanical Enhancement of Digestion. The best understood form of avian geophagy is the consumption of grit to enhance the mechanical grinding action of the muscular stomach. Many species of birds collect grit, particularly finches, geese, woodpeckers, corvids, and pipits (McIlhenny, 1932; Kilham, 1960; MacRoberts and MacRoberts, 1976; Verbeek, 1995). Best and Gionfriddo (1991) found that the mean grit size in 22 bird species foraging in cornfields varied

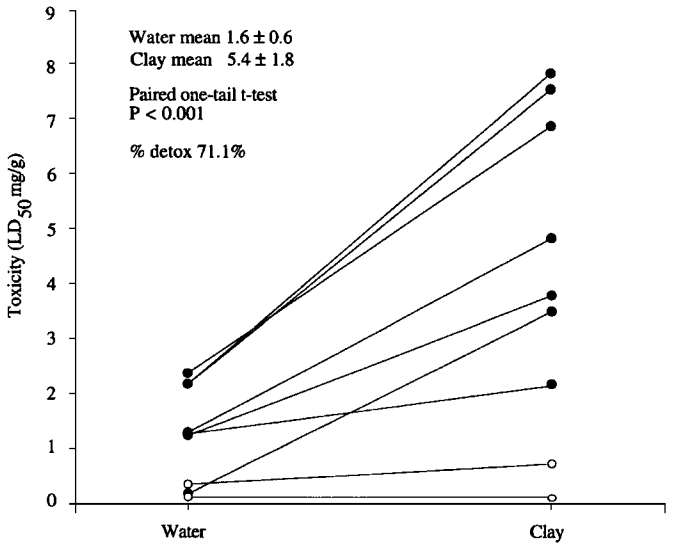

FIG. 6. Detoxification of seed samples by clay using a brine shrimp bioassay (means ± 2 SE). Sample identification from top to bottom on the clay data are unripe seeds of *Cedrela odorata*, *Hevea guianensis*, *Couratari guianensis*, *Spondias mombin*, whole ripe fruit of *Sapium marmieri*, *Annona* sp., unripe seeds of Qualea sp., and the hollow circles are standards of quinidine, digitonin.

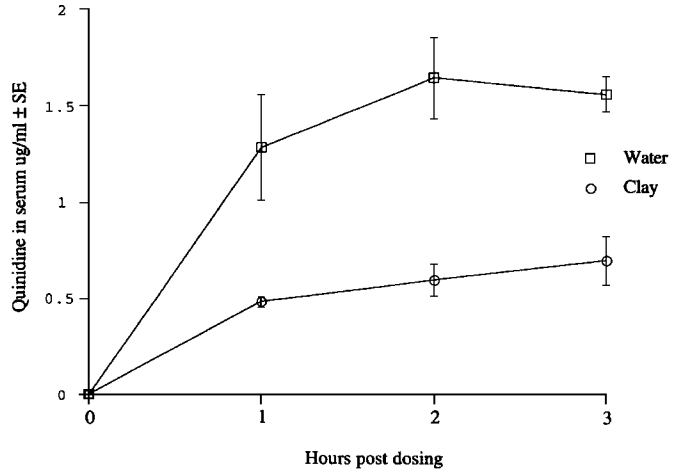

FIG. 7. Effect of clay ingestion on the bioavailability of quinidine in orange-winged Amazon parrots (*Amazona amazonica*).

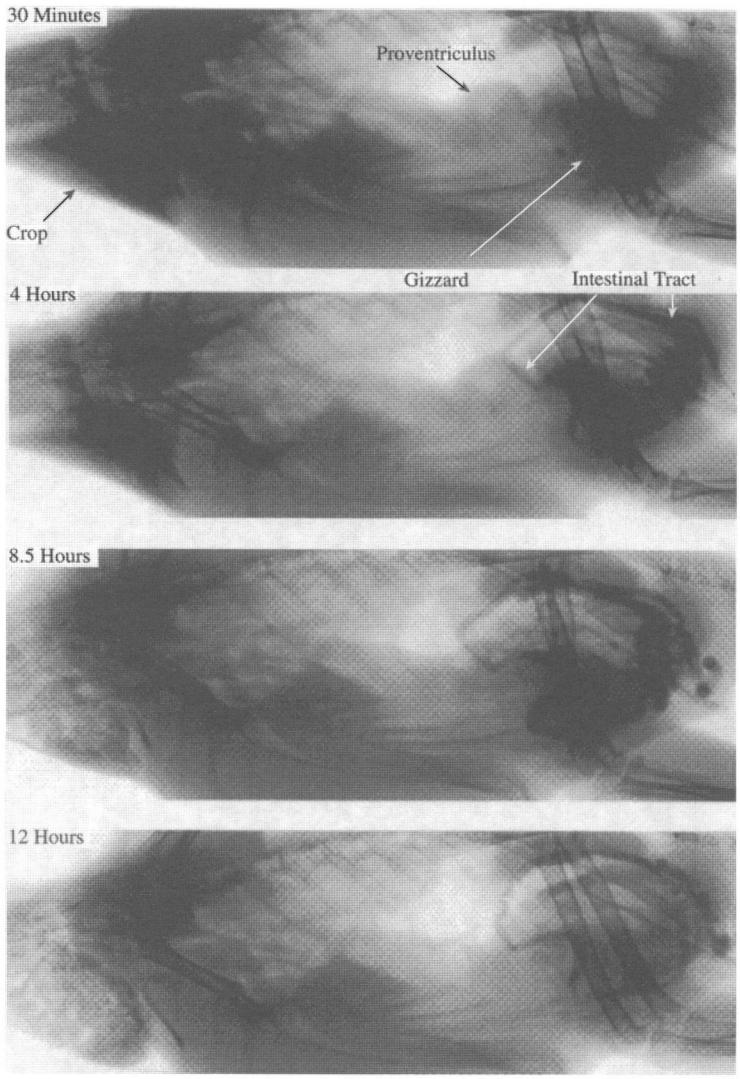

FIG. 8. Radiographs showing the distribution of labeled clay (a) and contrast agent alone (b) moving through the gastrointestinal tract of a blue-fronted Amazon parrot (*Amazona aestiva*).

from 0.5 to 3.5 mm and was directly correlated with body size. In contrast, we found that preferred soils of parrots in Peru were generally smooth in texture with a sand content mean of <5%, which strongly argues that birds do not eat soil to enhance the mechanics of digestion. Rather, the parrots choose fine-grained soils

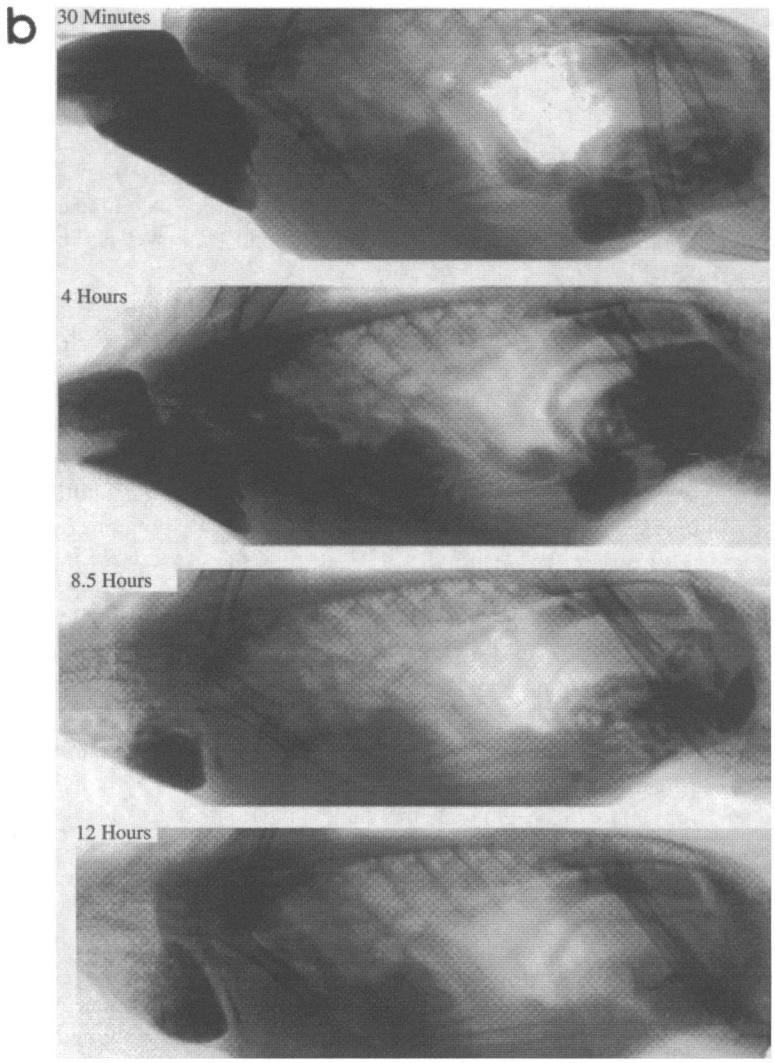

FIG. 8. CONTINUED.

with high clay content and correspondingly high cation exchange capacity (CEC) and presumably can adequately masticate hard food items with their powerful and dexterous bills. Hence, geophagy in parrots invites alternative hypotheses on its function based on the structure and potential function of the clay itself.

Mineral Supplementation. Clays, as crystalline minerals, have very high

surface areas and considerable potential to release minerals. Most reports of clay-eating in ungulates have concluded that animals consume soil for mineral supplementation of an otherwise deficient diet (cf. Jones and Hanson, 1985). Outside the scientific community, this assumption has led many to call geophagy sites salt-licks in English, collpas or salitrales in Spanish, and barrieros in Portuguese. A number of studies have looked at minerals present in soils from geophagy sites, and although the results vary among sites, sodium, calcium, and magnesium are among the most common minerals found (reviewed by Jones and Hanson, 1985).

There are three complicating issues, however, that many of these studies do not address: (1) total vs. available minerals, (2) mineral content of the diet, and (3) the effect of the ratio of gastric fluids to soil on the quantity of minerals released. First several studies in addition to ours suggest that not all minerals present in a soil sample are available and that a simulated gastric environment is a more meaningful way to determine what is available than the determination of "total minerals" (Hunter and De Kleine, 1984; Jones and Hanson, 1985). Second the availability of minerals in the animals' normal diets is frequently not considered, and in some cases exceeds the quantity of a given mineral(s) available in the soil. For instance, without the knowledge of minerals available in parrot diets, it might appear that these Peruvian soils contain biologically important and perhaps supplemental quantities of calcium, magnesium, iron, manganese, and sodium (Table 1). However, all but sodium are considerably more abundant in the animals' food resources than what is available in the soil. Third the ratio of the clay to extracting solution is important in vitro but is a difficult parameter to estimate in vivo. For instance, our in vitro results show that a bird consuming 5 g of soil might extract anywhere from 60 to 7500 μg of calcium, depending on the ratio of soil to gastric fluid as the soil passes through the gastrointestinal tract (Figure 2).

These considerations aside, much is known about geophagy in vertebrates and clearly minerals cannot be discounted altogether. There are several studies demonstrating attraction to minerals in ungulates, rabbits, and even butterflies (Blair-West et al., 1968; Arms et al., 1974; Jones and Hanson, 1985). From our data, we can rule out all the minerals except sodium as the primary cause of geophagy, based on the preferred/nonpreferred comparison, by evaluating the minerals in the birds' diets, or both. Although not significantly more available in the preferred soils, sodium is generally more available in the Peruvian soils than in the plant tissues they consume. In comparison with ungulate licks in North America, two of the preferred soils would be greater than Jones and Hanson's criteria for a lick "high in extractable sodium" (5 meq/100 ml $\approx$ 1150 ppm, 1985, p. 97), and two would be less than this figure.

However, five lines of evidence suggest that there is more to parrot geophagy than attraction to sodium. First, the sites vary widely in the quantity of available sodium. Some of the soils eaten by parrots in Tambopata contain

very little sodium, less than that in some tap water. This suggests that these birds are attracted to soil even if it contains little sodium. Second, we have found evidence that macaws in Tambopata feed on insect larvae (*Hermetia:* Stratiomyidae; Gilardi and Munn, unpublished data). Insect-eating by parrots has been observed in a variety of species in the Neotropics and in Australia (McInnes and Carne, 1978; Roth, 1984; Wyndham and Cannon, 1985; Sazima, 1989; Sick, 1993; Martuscelli, 1995) and presumably provides a source of protein, lipid, potassium, and sodium. By enhancing their sodium intake through insect foraging, parrots could avoid the huge congregations of birds that attract predators at the clay lick (Robinson, 1994; Munn, unpublished data), as well as the metabolic cost of returning from the clay lick with a full crop of soil having no caloric content. Third, we have offered these Peruvian soils to three parrot genera in captivity (*Amazona, Pionus, Trichoglossus*), and all consume it readily despite the fact that their commercial diets contain more sodium than any of the soils (>2000 ppm; Gilardi unpublished data). Fourth, experiments with budgerigars (*Melopsittacus undulatus*) and other seed-eating birds have shown that, while these birds avidly consume salt, they do not detect it until the concentration is greater than 3000 ppm, which is more than twice the highest value for these soils (Cade, 1964). Fifth, in ungulates where the evidence is strongest for mineral attraction, feeding trials have demonstrated increased growth rates of cattle and lambs when clay is added to a balanced diet and further increases in growth rates when the clay is available ad libitum (Burkitt, 1969; Mendel, 1971; Huntington et al., 1977). These observations suggest that the clay plays a role in digestion other than, or perhaps in addition to, release of minerals.

Buffering Capacity. Although the possibility that soils may buffer the pH of portions of the gastrointestinal tract has been suggested (Oates, 1978), the Peruvian soils show essentially no potential to alter pH. Additional testing of this mechanism in vivo may reveal some activity of the clay that our simple system fails to detect.

Adsorption of Dietary Toxins. Given the clay content of the Peruvian soils, the types of clays present, and the ability of clays to exchange cations, geophagy may provide the vertebrate consumer with two types of advantages: attenuation of toxicity by adsorption of toxins, and gastrointestinal cytoprotection from chemical insults to the gut lining. Several studies of geophagy in primates and humans (Hladick and Gueguen, 1974; Mahaney et al., 1996) have suggested that soils might adsorb dietary toxins, but this function of geophagy in primates remains to be tested in vivo. In humans, clay has been demonstrated to adsorb secondary compounds prior to consumption in the process of preparing acorns and semidomesticated potatoes (Johns, 1986; Johns and Duquette, 1991). Adsorption of, or protection from, secondary compounds would be particularly beneficial to macaws and other parrots that eat foods that are often quite toxic and contain high levels of phenolics (Gilardi and Munn, unpublished data).

Our in vitro adsorption trials show that detoxification via this mechanism is dependent on chemical identity; the soils were able to adsorb large quantities of the alkaloid quinine and relatively little of the polyphenolic tannic acid. Since polyphenolic content can be quite high in parrot diets (≈5% dry weight), the quantities of clay required to significantly reduce toxicity would be many times the weight of the birds' daily food requirements. However, for charged compounds such as alkaloids, which typically occur at much lower levels (<1% dry wt), and which have greater affinity for clays (≈100 mg adsorbed/g, Figure 5), consumption of several grams of soil per day could adsorb biologically significant quantities of the toxin. The clays also significantly reduced the toxicity of seeds that these birds normally consume, further supporting the possible role of clays in dietary detoxification.

Perhaps most importantly, our work with captive parrots demonstrates that geophagy can substantially reduce the bioavailability of a readily adsorbed alkaloid (quinidine) in a controlled experiment. In concert, these results strongly support the proposed function of geophagy as a means of detoxification of dietary toxins. Further tests of this mechanism might use a diet spiked with an easily monitored toxin and would ideally determine if animals can facultatively respond to dietary toxins by voluntarily increasing soil consumption. Such behavioral responses are well known in rats and are perhaps the clearest indication that some vertebrates respond to ingested poisons and nausea by eating soil (Mitchell et al., 1976; Takeda et al., 1993).

If geophagy does play a role in dietary detoxification, the benefits for parrots and other soil-eating vertebrates would be multiple. Geophagy would allow the bird or mammal to consume previously unexploitable resources and/or increased quantities of seeds and fruits that would otherwise cause illness or death. Furthermore, by reducing the exposure of the consumer to toxins, geophagy would reduce the costs of constitutive and inducible enzymatic detoxification (Berenbaum and Zangerl, 1994). Parrots are able to consume nutritionally rich but highly toxic resources during the dry season when food is limiting to other frugivores (Terborgh, 1986; Gilardi and Munn, unpublished data). Thus, by facilitating the expansion of the diet, geophagy may extend the distribution and/or enhance the local abundance of parrots and other animals. Similar benefits have been attributed to the consumption of charcoal by Zanzibar red colobus monkeys (*Procolobs kirkii*), which occur at elevated densities where this behavior occurs (Struhsaker, 1997).

Gastrointestinal Cytoprotection. Geophagy may serve yet another function resulting from the interaction of some clay types with the gut lining and the induction of a cytoprotective effect. This effect has been demonstrated in rats, rabbits, dogs, and humans and is particularly well studied in research on antidiarrheal medications (Rateau et al., 1982; More et al., 1987; Grandjean et al., 1992; Vivatvakin et al., 1992; Gardiner et al., 1993). Although the mechanism

of this interaction is poorly understood, the presence of clay in the gut increases mucus secretion by goblet cells and prevents mucolysis through increased protein cross-linking. The clay thereby enhances the ability of the mucus barrier to protect the gut lining from various chemical insults (Droy-Lefaix et al., 1985; Leonard et al., 1994). Clay also remains in the gut bound to the mucus layer, where it may continue to adsorb various toxins making the two functions, cytoprotection and adsorption, mutually compatible (Brouillard and Rateau, 1989; Gardiner et al., 1993; Leonard et al., 1994). Our findings on the passage rate of clays in captive parrots indicate that large amounts of clay were present in the gastrointestinal tract for at least 12 h after consumption, suggesting that relatively long-term exposure of the gastrointestinal mucosa occurs in parrots.

Recent studies have demonstrated that high surface area clays such as smectite and attapulgite are effective at inducing these cytoprotective effects and alleviating the symptoms of diarrhea (Leber, 1988; Lexomboon et al., 1994). As a result, the active ingredient of some antidiarrheal medications has been changed to these clay types from the historic kaolin–pectin formulation (Dukes, 1990). Soils from many geophagy sites are kaolin-rich (Vermeer and Ferrell, 1985; Mahaney et al., 1996), but it remains unclear whether these low-surface-area clays can induce cytoprotection. The Peruvian soils we tested contained moderate to high surface area clays, suggesting that consumption of these soils is likely to generate the cytoprotective effects induced by modern clay-based antidiarrheal medications.

Polyphenolics are known to attack and erode the lining of the gut, i.e., tanning the proteins in the mucus or on the mucosal surface, causing the types of damage that clay treatment can prevent (Mitjavila et al., 1977; Freeland et al., 1985; Mole and Waterman, 1986; Gee and Johnson, 1988; Ortiz et al., 1994). Although parrots generally do not exhibit the symptom of diarrhea (Lumeij, 1994), animals suffering the acute effects of large quantities of tannin-rich plants are often diagnosed with diarrhea and/or gastric lesions (Weisman and Thompson, 1984; Holliman, 1985; Hwang et al., 1991). Given that these parrots consume a diet that is high in phenolics, they may be protected from the corrosive effects of these compounds, but in this case, the mechanism involves the induction of a cytoprotective effect in the lining of the gastrointestinal tract. How this cytoprotective mechanism might relate to cation-exchange detoxification remains a question for future study, but there is no obvious theoretical reason why they might not function simultaneously.

In summary, analyses of geophagy soils and experiments on captive parrots strongly reject the grit and pH-buffering hypotheses, and although minerals are released, our data suggest that minerals are unlikely to be the primary cause of geophagy in parrots. From the in vitro adsorption trials, the effects on the toxicity of parrot food items, and the reduction of bioavailability of quinidine in captive birds, we conclude that geophagy can function to detoxify dietary toxins

for vertebrate herbivores. The persistence of clay in the gastrointestinal tract and the types of clay present in geophagy soils further suggest the possibility that cytoprotection of the gastrointestinal tract may also be an important function of geophagy. Since detoxification is likely to occur in the lumen of the gut and the gastrointestinal mucosa is roughly similar among vertebrates (Fawcett, 1994), these two functions, dietary detoxification and cytoprotection, may well be universally applicable to all soil-eating animals including humans, nonhuman primates, ungulates, and other herbivores. Because of their structures, however, soils can, and likely do, perform a variety of functions for vertebrate consumers. Given the complexities of plant chemistry, gastrointestinal physiology, and animal ecology, the causes of this phenomenon are likely to be multifactorial.

We propose three ecological adaptations that enable parrots to cope with toxic resources. First, a defining characteristic of the Psittaciformes is the exceptionally large, powerful, and manipulative bill. Macaws in particular are capable of opening the hardest of seeds, delicately peeling away chemically defended seed coats, and consuming the high-quality seed (Gilardi and Munn, unpublished data). Such handling of foods is common in herbivores, but is particularly well known in humans, where processing raw foods by peeling, leaching, boiling, baking, etc., is often a form of detoxification (cf., Stahl, 1984; Johns, 1990). Second, although exposure to household toxins often causes morbidity and mortality in captive parrots (Dumonceaux and Harrison, 1994), wild parrots frequently consume seeds that are toxic to humans, suggesting that parrots are capable of effective inducible enzymatic detoxification (Acedo, 1992; Munn, 1988; Gilardi and Munn, unpublished data; Norconk et al., 1997). Our finding that captive amazons required 50 times the human dose of quinidine to attain similar circulating levels of the drug supports the hypothesis that parrots avoid absorbing ingested toxins, detoxify them rapidly, or both. Finally, widespread and frequent geophagy of clay-rich soils may adsorb significant quantities of dietary toxins and prevent gastric erosion, thus facilitating consumption of otherwise poisonous foods.

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ANTIFUNGAL ACTIVITY OF SOME B,D-SECO LIMONOIDS FROM TWO MELIACEOUS PLANTS

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Abstract—Isolation and characterization of B,D-seco limonoids from *Swietenia mahogani* have been described. Seven limonoids from *S. mahogani* and five limonoids from *Khaya senegalensis* were tested for antifungal activity against the groundnut rust *Puccinia arachidis*. Mexicanolide, 3β-acetoxy-mexicanolide, 3β-hydroxymexicanolide, 2α,3β-dihydroxymexicanolide from *K. senegalensis*, and 6-acetylswietenine and 6-acetyl-3-tigloylswietenolide from *S. mahogani* effectively reduced the number of rust pustules on detached groundnut leaves. Possible correlation of structure to antifungal activity is discussed.

Key Words—Limonoids, *Swietenia mahogani*, *Khaya senegalensis*, *Puccinia arachidis*, antifungal activity.

INTRODUCTION

Triterpenoids are an important group of constitutive defense substances present at sufficient concentrations to ward off potential plant pathogenic fungi (Grayer and Harborne, 1994). Triterpenoids from the family Meliaceae, in particular, are highly diversified in structure and have been studied extensively for their insect antifeedant and growth regulating activities (Champagne et al., 1992). Extracts from seeds of the neem tree *Azadirachta indica* containing triterpenoidal compounds are known to be effective against plant pathogenic fungi (Khan et al., 1974; Singh et al., 1980; Steinhauer, 1995; Locke, 1995; Coventry and Allan,

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1996; Govindachari et al., 1998). Antifungal triterpenoids of the Meliaceae include four meliacins from *Chisocheton paniculatus* (Bordoloi et al., 1993) and nimonol and isomeldenin from *Azadirachta indica* (Suresh et al., 1997).

A number of limonoids have been reported from the genus *Swietenia* (the true mahoganies) with structures assigned on the basis of spectral data (Kadota et al., 1990). All of these have been isolated by conventional column chromatography, with different adsorbents, eluents, and work-up procedures (Okorie and Taylor, 1970; Chan et al., 1976; Connolly et al., 1965, 1968; Rao et al., 1978; Taylor and Taylor, 1983). In this paper, we report the isolation of seven limonoids from *S. mahogani* by direct preparative high-performance liquid chromatography (HPLC) of a fraction obtained from the methanolic extract of the seeds. *Khaya senegalensis* also elaborates B,D-seco limonoids (Adesida et al., 1971). Isolation of several of these compounds by HPLC has been reported (Govindachari et al., 1997). We evaluated the triterpenoids from *S. mahogani* and *K. senegalensis* for their antifungal activity by using the groundnut rust *Puccinia arachidis*.

METHODS AND MATERIALS

Extraction of B-seco Limonoids from Swietenia mahogani. Seeds of *Swietenia mahogani* were collected (from the western ghats, Tamil Nadu, India) and identified by Dr. K. V. Krishnamuthy (a voucher specimen is available at the Department of Botany, Bharathidasan University, Trichy, Tamil Nadu). Details of the extraction of seed kernels are given in Figure 1.

Isolation of B,D-seco Limonoids from Swietenia mahogani. The residue of fractions 27–45 (Figure 1) from medium pressure liquid chromatography (MPLC) (8.7 g) was subjected to HPLC for isolation of major limonoids. For each preparative run, 400 mg of the residue was dissolved in 4 ml MeOH, filtered through a Millipore filter (0.22 μm), and then injected into the column (Shimpack C-18, 25 cm × 20 mm). Acetonitrile–methanol–water (40 : 20 : 40) was used as eluent at a flow rate of 10 ml/min throughout the run. Peaks 1–7 (Figure 2) were collected individually and evaporated. The residues obtained were subjected to subsequent column chromatography (gravity/Lobar) to obtain pure limonoids. Lichroprep Si 60 (40–63 μm) [Merck, Lobar column size B (310–351)] was used for Lobar column chromatography.

Column chromatography (gravity) of HPLC peak 1 (31.98 min, 290 mg) and peak 2 (35.68 min, 530 mg), after elution with 60 : 40 hexane–ethyl acetate (EtOAc), yielded swietenine (48 mg) and 3-tigloylswietenolide (85 mg). The residue from HPLC peak 3 (40.54 min, 1.3 g) was subjected to chromatography with a Lobar column (Merck) (5 : 95 ethyl acetate–chloroform), and yielded 3,6-di-*O*-acetylswietenolide (41 mg). HPLC peak 4 (51.52 min, 678 mg) showed a number of closely moving spots in TLC (8 : 92 ethyl acetate–chloroform)

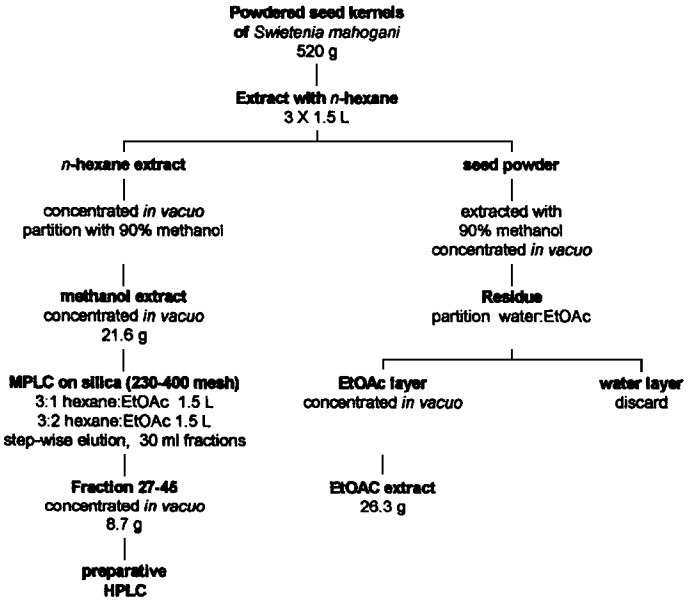


FIG. 1. Extraction of limonoids from the seed kernels of *Swietenia mahogani*.

and was not further investigated. Lobar column chromatography of the residues obtained from peak 5 (63.33 min, 1.4 g), peak 6 (73.02 min, 855 mg), and peak 7 (83.19 min, 311 mg) with 8 : 92, 5 : 95, and 2 : 98 ethyl acetate–chloroform as eluents yielded 6-acetylswietenine (32 mg), 6-acetyl-3-tigloylswietenolide (33 mg), and 6-desoxyswietenine (40 mg).

The EtOAc extract (derived from the methanol extract of the seed kernels) (8 g) was subjected to gravity column chromatography (60–120 mesh Si gel) with hexane as the eluent, and the polarity was slowly increased to pure ethyl acetate. Fractions 57–70 obtained from 1 : 1 hexane–ethyl acetate were mixed together based on TLC to give a residue of 2.3 g. This residue was then subjected to a Lobar column chromatography (1 : 1 hexane–ethyl acetate). Fractions 7–10 were pooled and chromatographed on a Lobar column with 40% ethyl acetate in chloroform. Fractions 1–19 were pooled to give swietenine (166 mg), and fractions 22–26 were pooled to give swietenolide (59 mg). Swietenine [mp 198–201°C, MF $C_{32}H_{40}O_9$(M^+568)], 3-tigloylswietenolide [mp 194–197°C, MF $C_{32}H_{40}O_9$ (M^+568)] 3,6-di-*O*-acetylswietenolide [mp 154–155°C, MF $C_{31}H_{38}O_{10}$ (M^+570)], 6-acetylswietenine [mp 130–132°C, MF $C_{34}H_{42}O_9$ (M^+594)], 6-acetyl-3-tigloyl swietenolide [mp 116–120°C, MF$C_{34}H_{42}O_9$ (M^+594)], 6-desoxyswietenine (febrifugin) [mp 186–189°C, MF $C_{32}H_{40}O_8$ (M^+552)], and swietenolide [mp 154–158°C, $C_{27}H_{34}O_8$ (M^+486)]

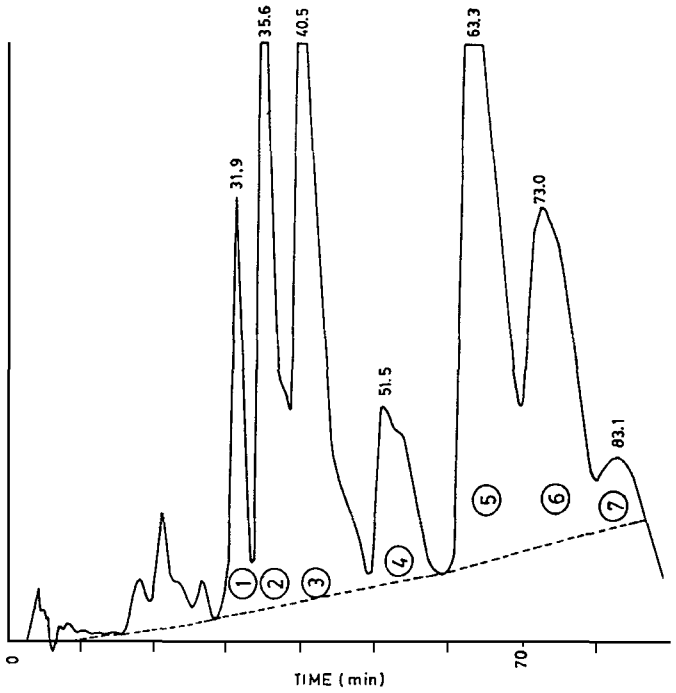


FIG. 2. Preparative high performance liquid chromatogram of the residue from *Swietenia mahogani* seed kernels (fractions 27–45 from medium pressure liquid chromatography).

were identified by comparison of the ^{1}H and ^{13}C NMR data with reported values. Structures are presented in Figure 3.

Isolation of B-seco Limonoids from Khaya senegalensis. Powdered seeds (1.015 kg) of *K. senegalensis* (collected from the social forests of Maharashtra state, India) were extracted exhaustively with *n*-hexane and the defatted seed powder reextracted with ethanol. The ethanol extract, after removal of solvent, was partitioned between EtOAc and water. The EtOAc layer was concentrated in vacuo. The residue (50.4 g) was subjected to vacuum liquid chromatography, and the eluting solvent was hexane with increasing quantities of EtOAc (10–50%), and then 100% EtOAc. Fractions that eluted with 30 and 40% EtOAc in hexane (9.5 g) gave mexicanolide (1.5 g). The pure EtOAc fraction was concentrated to dryness (10.5 g) and subjected to HPLC (Govindachari et al., 1997). Reverse-phase preparative HPLC with a RP18 (25 cm × 20 mm) column with methanol–water (60 : 40) as eluent at 10 ml/min flow (detection at 215 nm) yielded 2α-hydroxymexicanolide (R_t 27.37 min, 150 mg), 2α,3β-dihydroxymexicanolide (R_t 50.6 min, 100 mg), and 3β-hydroxymexicanolide (R_t 74.4

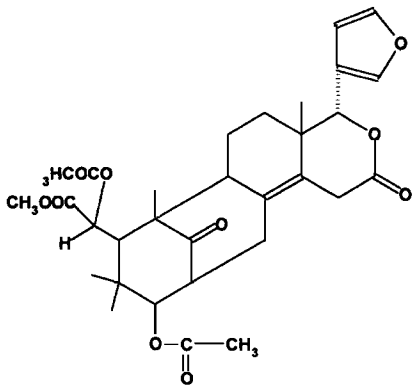
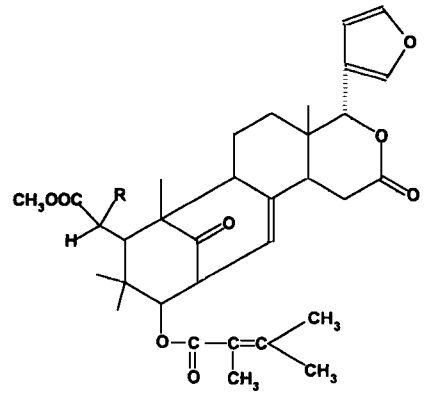


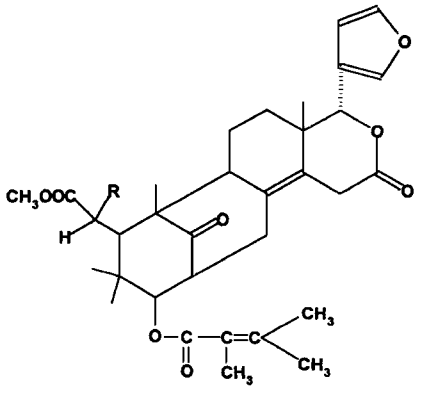
FIG. 3. B,D-seco limonoids used in this study.

min., 945 mg). 3β-Acetoxymexicanolide was isolated from the methanol wash of the *n*-hexane extract by HPLC. For spectral details see Govindachari and Krishna Kumari (1997).

Antifungal Bioassay. The bioassay used was that of Suresh et al. (1997). *Arachis hypogaea* L. var. TMV 2 was raised and maintained in a number of 3- × 3-m plots. Detached leaflets of 60-day-old groundnut plants were infected with 0.1 ml of a field-collected uredospore suspension of *Puccinia arachidis* Speg. (ca. 50,000 spores/ml) by spreading the spore suspension on the abaxial surface of the leaflet. The infected leaflets were kept in Petri dishes covered inside with moistened filter paper to maintain humidity. For the antifungal bioassay, compounds were dissolved in acetone to obtain concentrations of 0.1, 1, and 10 $\mu g/cm^2$ of leaflet area and spread evenly on the abaxial surface. Acetone alone



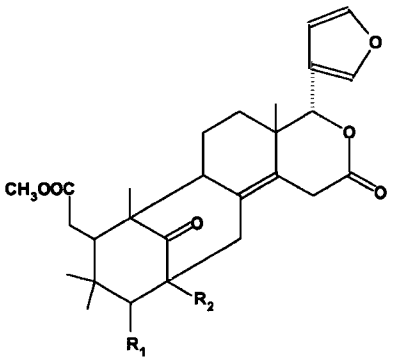
R = OH Swietenine
R = H Febrifugin
R = $OCOCH_3$ 6-acetylswietenine



R = OH 3-tigloylswitenolide
R = $OCOCH_3$ 6-acetyl-3-tigloylswietenolide

FIG. 3. CONTINUED.

served as a solvent control. After air-drying, 0.1 ml of the spore suspension was spread evenly on the abaxial surface, and subsequently the leaflets were kept in the Petri dishes. Twenty replicates were maintained for control and for each concentration and compound. The Petri dishes were kept in a dark chamber for 48 hr at 24 ± 2°C to facilitate infection and subsequently were transferred to a light chamber (3000 lux). The number of rust pustules appearing on day 12 were counted on each leaflet. Percent reduction in rust pustules over the respective control leaflets were calculated by the formula



$R_1 = O$ $R_2 = OH$ 2α-hydroxymexicanolide
$R_1 = OH$ $R_2 = OH$ 2α,3β-dihydroxymexicanolide
$R_1 = OH$ $R_2 = H$ 3β-hydroxymexicanolide
$R_1 = OAc$ $R_2 = H$ 3β-acetoxymexicanolide
$R_1 = O$ $R_2 = H$ mexicanolide

FIG. 3. CONTINUED.

$$100 - \frac{\text{number of rust pustules in treated leaflet}}{\text{number of rust pustules in control leaflet}} \times 100$$

The data (percent reduction in pustule number for each replicate of the treatment) were subjected to analysis of variance (ANOVA) (completely randomized blocks) by using a COSTAT program in an IBM PC/AT computer. Newman-Keul means and ± SE (0.05) were calculated and are presented in Table 1.

RESULTS AND DISCUSSION

We previously established the utility of preparative HPLC for the isolation of limonoids (Govindachari et al., 1995, 1996, 1997). That the tetranortriterpenoids can be separated by preparative HPLC has been confirmed in the present investigation by the separation of semipure B-seco limonoids from the seeds of *S. mahogani*. B-seco limonoids of the mexicanolide type from the seeds of *K. senegalensis* have been isolated previously by preparative HPLC (Govindachari et al., 1997). The compounds isolated from *S. mahogani* were reported previously; however, 6-desoxyswietenine has been isolated from *S. mahogani* for the first time. It was previously reported as febrifugin, from *Soymida febrifuga* (Meliaceae) by Rao et al. (1978).

Among the 12 limonoids tested for antifungal activity, 6-acetylswiete-

TABLE 1. REDUCTION IN RUST PUSTULE EMERGENCE ON DETACHED GROUNDNUT LEAVES TREATED WITH B,D-SECO LIMONOIDS[a]

Compound	Emergence reduction (%)		
	0.1 $\mu g/cm^2$	1 $\mu g/cm^2$	10 $\mu g/cm^2$
3,6-Di-*O*-acetyl swietenolide	36.4 ± 11.3bc	35.5 ± 20.7ab	63.6 ± 11.7ab
Swietenolide	3.6 ± 18.1b	−3.3 ± 25.6a	65.4 ± 8.0ab
6-Acetyl swietenine	74.9 ± 9.9c	80.4 ± 3.7b	84.8 ± 3.3b
6-Acetyl-3-tigloyl swietenolide	69.5 ± 8.8c	30.9 ± 24.4ab	84.7 ± 3.6b
Febrifugin	−54.0 ± 35.5a	47.7 ± 12.1b	37.6 ± 17.1a
Swietenine	−99.0 ± 53.4a	−186.5 ± 70.1a	−42.6 ± 52.7a
3-Tigloylswietenolide	−67.1 ± 71.1a	25.0 ± 29.1b	8.9 ± 30.7a
2α,3β-Dihydroxymexicanolide	80.0 ± 5.6b	90.2 ± 3.6b	94.8 ± 4.6b
3β-Acetoxymexicanolide	80.3 ± 7.7b	80.8 ± 5.4b	89.4 ± 2.5b
3β-Hydroxymexicanolide	−23.3 ± 47.1ab	81.2 ± 6.5b	89.4 ± 2.8b
Mexicanolide	13.6 ± 56.6b	59.3 ± 16.8b	76.6 ± 7.9b
2α-Hydroxymexicanolide	−107.4 ± 57.4a	−114.2 ± 69.9a	51.8 ± 16.6a

[a]Values indicate Neumann-Keul means ± SE. Values followed by the same letter are not significantly different.

nine, 6-acetyl-3-tigloylswietenolide (from *S. mahogani*), 2α,3β-dihydroxymexicanolide, 3β-hydroxymexicanolide, 3β-acetoxymexicanolide, and mexicanolide (from *K. senegalensis*) effectively reduced rust pustule emergence, thereby reducing disease severity. At 10 $\mu g/cm^2$ leaflet area, over 80% reduction in pustule number was observed in treated leaflets. Surprisingly, while 3,6-di-*O*-acetylswietenolide and swietenolide were moderately active, swietenine and 2α-hydroxymexicanolide increased disease severity considerably at lower concentrations relative to controls. A literature search indicated that information on antifungal activity of limonoids is restricted to three reports pertaining to either intact apo-euphol limonoids or C-seco limonoids from the Meliaceae (Bordoloi et al., 1993; Suresh et al., 1997; Govindachari et al., 1998). This report shows that some of the B,D-seco limonoids also effectively reduce groundnut rust disease severity. Swietenine and its derivatives have a characteristic 8,30 double bond, while swietenolide and mexicanolide-type compounds have an 8,14 double bond. Other than the double bond position, the individual compounds differed in the substitutions at the C-2, C-3, and C-6 positions.

Comparisons of swietenine with 3-tigloylswietenolide and 6-acetylswietenine with 6-acetyl-3-tigloylswietenolide indicate clearly that the double-bond position does not influence the antifungal activity. Increased activity of the latter two compounds, however, suggests that functional group substitutions may be important. Furthermore, the comparison between swietenine and 6-acetylswietenine on one hand and 3-tigloylswietenolide and 6-acetyl-3-tigloyl swietenolide on the other reiterates that substitution at C-6 is important. Compounds possess-

ing an acetoxy group at C-6 reduce mean pustule numbers per leaflet, while compounds such as febrifugin, swietenine, and 3-tigloylswietenolide (the former two having an OH at C-6 and the latter unsubstituted at C-6) increase disease severity at lower concentrations. At higher concentrations (10 $\mu g/cm^2$ leaflet area), these compounds lack any appreciable antifungal activity. These results confirm earlier observations that substitution at C-6 is crucial for antifungal activity.

3,6-Di-*O*-acetylswietenolide and swietenolide reduce pustule number per leaflet at 10 $\mu g/cm^2$ (by up to 65%) but at lower concentrations have no appreciable activity. Substitution of an acetyl group at C-3 (as in 3,6-di-*O*-acetylswietenolide) instead of a tigloyl (as in 6-acetyl-3-tigloylswietenolide) results in reduced antifungal activity. Swietenolide, having a C-3 hydroxyl, retained moderate antifungal activity.

Mexicanolide derivatives such as 2,3-dihydroxymexicanolide, 3-hydroxymexicanolide, and 3-acetoxymexicanolide showed excellent activity, reducing pustule number per leaflet over 80% at 10 $\mu g/cm^2$. Mexicanolide (having a C-3 carbonyl function), although antifungal at 10 $\mu g/cm^2$, had limited or no activity at lower concentrations. 2α-Hydroxymexicanolide (with α-hydroxyl substitution at C-2 and a carbonyl function at C-3) increased disease severity considerably at lower concentrations. At 10 $\mu g/cm^2$, it decreased pustule number up to 42%.

A number of Meliaceae triterpenoids have been shown to be insect antifeedant compounds, while a few are antifungal as well (Bordoloi et al., 1993; Govindachari et al., 1998; Suresh et al., 1997). It seems probable that such compounds are chemical defenses against insects and fungi. The present investigation on B,D-seco limonoids from two meliaceous species has indicated that mexicanolide type compounds are effective against groundnut rust pathogen. The detached leaf technique and the groundnut rust pathogen is only a model assay system for rapid evaluation of the antifungal activity of triterpenoids (Suresh et al., 1997). It is unknown whether the constitutive accumulation of these compounds in the seeds of *S. mahogani* and *K. senegalensis* is directed against pathogenic fungi colonizing the plant species.

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ANTIFUNGAL DIKETOPIPERAZINES FROM SYMBIOTIC FUNGUS OF FUNGUS-GROWING ANT *Cyphomyrmex minutus*

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Abstract—The attine fungus *Tyridiomyces formicarum*, the symbiont of the fungus-growing ant *Cyphomyrmex minutus*, produces several antifungal diketopiperazines. This represents the first identification of antifungal compounds from an attine symbiont and contradicts previous suggestions that attine fungi do not produce metabolites with antifungal activity. *T. formicarum* probably produces antifungal compounds in defense (1) against other fungi that invade the gardens and escape the weeding activity of the ants, or (2) against ant-pathogenic fungi that could harm the host ants. Fungi cultivated by fungus-growing ants may represent a rich source of additional bioactive metabolites.

Key Words—Antifungal, diketopiperazine, *Cyphomyrmex minutus*, fungus-growing ant, symbiosis, *Tyridiomyces formicarum*

INTRODUCTION

Microorganisms in mutualistic associations with larger organisms are a rich source of secondary metabolites. The importance of these associations for the discovery of novel compounds is illustrated by two recent examples, the isolation of the anticancer drug taxol from an endophytic fungus of the Pacific yew (Stierle et al., 1993) and the isolation of the antiinflammatory depsipeptide salin-

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amide from an actinomycete inhabiting the surface of the jellyfish *Cassiopeia xamachana* (Trischman et al., 1994). Symbiotic organisms are particularly likely to produce biologically active compounds because of the following reasons; (1) The raison-d'etre for symbiosis frequently rests on the chemical benefits that symbionts derive from each other (Douglas, 1994). (2) Symbiotic organisms are strong ecological interactors and are thus subject to strong selection and rapid evolution (Futuyma and Slatkin 1983; Thompson, 1994). (3) Coevolution between mutualistic partners leads to the evolution of metabolites with specific modulatory effects (Futuyma and Slatkin, 1983). As part of a program to explore these "biorational" guidelines in the search for novel compounds (Eisner, 1990; Beattie, 1992), we screened the symbiont of the fungus-growing ant *Cyphomyrmex minutus* (Attini: Formicidae) for bioactive metabolites.

Fungus-growing (attine) ants depend on cultivated fungus for food in a mutualistic dependency that originated about 50 million years ago (Weber, 1972; Schultz and Meier, 1995). The attine fungus chosen in this study, *Tyridiomyces formicarum* (Wheeler, 1907), is cultured only by ants in the *Cyphomyrmex "rimosus"* group (Snelling and Longino, 1992; Schultz and Meier, 1995) and is unique among the attine fungi by growing as a yeast form (unicellular) and not as the mycelial form (multicellular) typical for all other attine fungi. Because of its unique growth form, *T. formicarum* had been thought to be distinct taxonomically from all other attine fungi (Wheeler, 1907; Weber, 1972, 1982). However, *T. formicarum* reverts to a hyphal growth form in artificial culture and then shows morphological similarity to other attine fungi (Hervey et al., 1977), suggesting that *T. formicarum* is merely a unicellular growth variant of the typical mycelial attine symbionts. Indeed, a recent phylogenetic analysis groups *T. formicarum* together with other attine fungi and places them near free-living lepiotaceous fungi in the genera *Leucoagaricus* and *Leucocoprinus* (Chapela et al., 1994; Mueller et al., 1998).

Several attine fungi had previously been screened for antibiotic activity (Martin et al., 1969; Weber, 1972; Hervey et al., 1977), and Hervey and Nair (1979) reported the isolation of the antibacterial lactol lepiochlorin from the symbiont of the ant *Cyphomyrmex costatus*. Attempts to demonstrate antifungal activity of extracts from attine fungi were negative (Martin et al., 1969; Weber, 1972), however, and contradicted sporadic observations on pure symbiont cultures that revealed growth inhibition against unspecified contaminant fungi (Weber, 1955). The absence of antifungal activity is surprising from an ecological perspective, because the symbiotic fungi are expected to coevolve with pathogenic and parasitic fungi that invade the nutrient-rich gardens, evade the weeding activity of the ants, and thus compete with the symbionts for resources. Based on this biorational analysis (Eisner, 1990), we began screening attine fungi for antifungal metabolites despite the previously reported failure (Martin et al., 1969; Weber, 1972).

METHODS AND MATERIALS

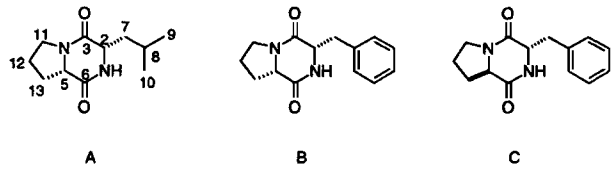
Colonies of the fungus-growing ants *Cyphomyrmex minutus* and *C. rimosus* were collected in March 1994 at the Archbold Biological Station, Lake Placid, Florida. Fungal isolations followed the methods described in Mueller et al. (1996). Individual nests (fungus garden and ants) were kept in containers for at least one week. This allowed ants to eliminate contaminants from gardens and thus minimized contamination during the fungal isolation. Single bromatia (clumps of yeast cells) were taken from gardens with a sterilized needle and transferred to acidified (pH 4.5–5.0) potato dextrose agar plates (PDA; Difco, Detroit, Michigan). Plates were monitored daily to eliminate fast-growing contaminants. Dense, fine mycelium typical for attine fungi covered uncontaminated inocula within one or two days. Isolates were taken from growth fronts after three weeks and transferred to PDA slants.

Preliminary agar-plug assays of fungal isolates from the ants *C. rimosus* and *C. minutus* revealed strong antifungal activity against the yeasts *Saccharomyces cerevisiae* and three strains of the human pathogen *Candida albicans* (ATTC strains C109, 406, and Wisconsin). Because fungi from *C. rimosus* and *C. minutus* are closely related (Chapela et al., 1994) and may in some cases represent recent descendants from the same fungal clone (Mueller et al., 1998), only one fungus from *C. minutus* (UGM940317-01) was chosen for further analysis. The fungus was grown at room temperature in potato dextrose broth supplemented with 0.5% tryptone (Difco). Liquid cultures were shaken at 200 rpm. Seven 1-liter flasks (600 ml broth/flask) were inoculated each with a small inoculum of the yeast symbiont and shaken for 14 days. The cultures were then filtered through Celite, and the filtrate was extracted twice with equal volumes of ethylacetate.

The crude extract (450 mg) was subjected to Sephadex LH-20 column chromatography and eluted with methylene chloride and methanol (1 : 1). Fractions were combined based on their TLC profile and tested for antifungal activities. The antifungal fraction (317 mg) was further fractionated on a reverse-phase flash column (C_{18}) and eluted with increasing amounts of MeOH in H_2O (0, 30, 50, 75, 100% MeOH). Reverse-phase (C_{18}) HPLC (19% MeCN in H_2O) of the antifungal fraction (153 mg) afforded three antifungal metabolites: compound A (6 mg), compound B (20 mg), and compound C (4 mg).

RESULTS

The high-resolution FAB mass spectrum of the compound A ($[M+H]^+$ 211.145) indicated the formula $C_{11}H_{18}N_2O_2$. DEPT, ^{13}C, and ^1H NMR data indicated the presence of two amide carbonyls, three methines, four methylenes, and



A B C

FIG. 1. Structures of the three antifungal compounds isolated from *Tyridiomyces formicarum*, the fungal symbiont of the fungus-growing ant *Cyphomyrmex minutus*.

two methyl groups. COSY data revealed $CHCH_2CH(CH_3)_2$ and $CHCH_2CH_2CH_2$ subunits. Long-range carbon–proton correlations were provided by HMBC data. Long-range carbon–proton correlations were provided by HMBC data. Long-range C-H correlations of a methylene (1.51/1.94/39.5 ppm, $C7H_2$) to two methyl doublets (0.95/22.3 and 0.96/23.4 ppm, $C9H_3$ and $C10H_3$) and a carbonyl carbon (169.1 ppm, C3) indicated a valine unit. The presence of a proline was confirmed by the unsaturation number and HMBC spectrum, which showed cross peaks of a methylene (2.02/2.30/29.2 ppm, $C7H_2$) to a carbonyl carbon (172.9 ppm, C6), and a methylene (3.50/46.6 ppm, $C11H_2$) to a carbonyl carbon (169.1 ppm, C3). Based on the unsaturation number and nitrogen count, the remaining atoms must be arranged to form a diketopiperazine ring leading to the structure shown in Figure 1 for compound A. Compound A presumably is derived from valine and proline.

The molecular formula of B was deduced as $C_{14}H_{16}O_2N_2$ from the high-resolution FAB mass spectrum ($[M+H]^+$ 245.129). DEPT, ^{13}C, and ^1H NMR data indicated the two amide carbonyls, a monosubstituted phenyl ring, two methines, and four methylenes. A methylene (3.15/38.3 ppm, $C7H_2$) shows long range C-H correlations to an amide carbonyl carbon (167.0 ppm, C3), two aromatic carbons (137.5 and 131.2 ppm), and a methine (4.44/57.8 ppm, C2), indicating the presence of a phenylalanine subunit. COSY and HMBC data also revealed a proline subunit. These results, along with the unsaturation number, allowed the structural assignment of compound B (Figure 1). The high-resolution FAB mass spectrum of compound C ($[M+H]^+$ 245.129) indicated the formula $C_{14}H_{16}O_2N_2$, identical to that of compound B. ^1H NMR, DEPT, ^{13}C, COSY, and HMQC data gave similar data and the same correlations. These data suggest that compound C is a stereoisomer—most likely an epimer—of compound B (Figure 1).

Acid hydrolysis of compounds A–C afforded the constituent amino acids. Preparation of the *N*-(pentafluoropropionyl)isopropyl ester derivatives, followed by chiral GC analysis on a Chirasil-Val column, showed that all of the amino acids from compounds A and B have the L absolute configuration. Compound C gave D-proline and L-phenylalanine, indicating that it is epimeric to compound C at the proline alpha carbon.

In a standard agar-diffusion assay against *Candida* 406 (see above), compound A caused a 9-mm zone of inhibition at 150 μg/disk, while compounds B and C gave 7-mm zones of inhibition at 300 μg/disk. In a positive control, Nystatin (100 units) induced a 19-mm zone of inhibition, indicating that compounds A–C are weakly antifungal.

DISCUSSION

The organic extracts of the attine fungus *Tyridiomyces formicarum* yielded three antifungal diketopiperazines (Figure 1) that are apparently derived from common amino acids (valine and proline). To our knowledge, this is the first identification of antifungal metabolites from an attine symbiont. The three isolated diketopiperazines showed only moderate antifungal activity in standard bioassays. The observed high antifungal activity of live cultures and extracts of *T. formicarum* suggests that the fungus employs a strategy of producing large amounts of a moderately potent mixture, rather than smaller amounts of more potent compounds. Indeed, the total amount of diketopiperazines produced by *T. formicarum* accounts for 9% of the weight of the crude extract, and compound B alone constituted almost 5% of the crude extract.

Diketopiperazines are widespread bioactive compounds of fungi that have been isolated from deuteromycetous, ascomycetous, and basidiomycetous fungi (Chen, 1960; Kodaira, 1961; Trigos et al., 1995; Arnone et al., 1996; Kozlovskii et al., 1997), and stereoisomeres of the compounds obtained from *T. formicarum* had been isolated previously from other fungi (Chen, 1960; Kodaira, 1961). The production of diketopiperazines by attine fungi therefore is not surprising and may indicate a general antifungal defense, rather than a specialized defense against particular kinds of fungal competitors. Fungus gardens present a rich resource base for parasitic and contaminant fungi, and the symbiotic fungi therefore are likely to compete with many types of alien fungi that escape the weeding activities of the ants. The high levels of antifungal diketopiperazines may therefore suppress or eliminate such fungal competitors in the garden. Alternatively, and not exclusive with the first hypothesis, antifungal compounds of the symbiont may serve a sanitary function and may protect the ant hosts against ant-pathogenic fungi, as has been hypothesized by Kermarrec et al. (1986) for leaf-cutting ants. Additional functions of the symbiont diketopiperazines cannot be excluded at this point. For example, fungal diketopiperazines have been shown to possess a variety of properties, including not only antifungal properties, as discussed above, but also antibacterial (Arnone et al., 1996) and antiviral properties (Tomassini et al., 1996). Diketopiperazines therefore may also serve additional functions in the symbiosis between ants and their cultivated fungi.

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OVIPOSITION STIMULANT FOR A GALL-INDUCING SAWFLY, *Euura lasiolepis*, ON WILLOW IS A PHENOLIC GLUCOSIDE

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Abstract—The ovipositional stimulation for the shoot galling sawfly, *Euura lasiolepis*, whose natural host is the North American willow, *Salix lasiolepis*, was studied in response to its original host, Finnish willows, poplar species, and individual phenolic glucosides and fractions. The major phenolic glucosides in the natural host were salicortin and tremulacin, which were also the major components in the Finnish *S. purpurea* and *S. rosmarinifolia*. The natural host, *S. lasiolepis*, was chosen over six Finnish willow species in multiple-choice tests. Multiple-choice tests with six Finnish willow species and one Finnish popular species showed that *S. rosmarinifolia* was significantly more attractive than any of the other species by at least 16-fold. No-choice testing with phenolic glucosides showed the tremulacin fraction to be the significant ovipositional stimulant, which elicited probes with the ovipositor at least 19 times more frequently than other glucosides. A tremulacin fraction at 1.5% elicited significantly more probes than a tremulacin fraction tested at 0.4%. In addition, tremulacin was the only glucoside to induce prolonged and repeated probing with the ovipositor, lasting a mean of 6.4 sec per bout per female. Only single probes lasting a fraction of a second were observed in response to other glucosides. This is the second study showing a direct effect of a phenolic glucoside as an ovipositional stimulant for a sawfly, and it also suggests the existence of a mechanism that enables females to respond positively in a graded way to shoot length heterogeneity in host plants and populations.

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Key Words—*Euura lasiolepis*, galling sawfly, host selection, Hymenoptera, insect herbivore, oviposition stimulant, phenolic glucosides, Salicaceae, *Salix*, Tenthredinidae.

INTRODUCTION

The chemical ecology of gall-inducing sawflies is poorly known. No sex pheromones have been isolated or identified, and only one defensive compound has been identified (Boevé et al., 1984), although repellency may be minimal (Boevé and Pasteels, 1985). In fact, during the development of the field of chemical ecology, the gall-inducing sawflies have played no role. This situation contrasts with developments in chemical ecology on the free-feeding sawflies (e.g., Codella and Raffa, 1993; Eisner, 1994).

Nevertheless, studies on the ecology of some gall-forming sawflies have revealed their very close ties to specific host-plant species in the Salicaceae, and high precision in many species while selecting shoots for oviposition (Price and Roininen, 1993; Price et al., 1995). Particularly in the genus *Euura*, which forms galls on stems, petioles, buds, and leaf midribs, we have repeatedly detected a strong female ovipositional preference for long shoots that are in the rarest shoot-length-class categories. This behavior is linked to higher survivorship of eggs and larvae in the longer shoots, making these kinds of sawflies some of the best examples known to high preference–performance linkage between female oviposition and larval survival (e.g., Craig et al., 1989; Price et al., 1995). Thus, we have an evolutionary or ultimate mechanistic explanation of why females prefer long shoots for oviposition. Natural selection has resulted from higher progeny survival in longer shoots feeding back positively on those females with the strongest ability to detect long shoots.

Lacking, however, has been a proximate mechanistic explanation for the ability of females to detect long shoots. We found that total phenolics in shoots were poor predictors of sawfly attack in the species *Euura lasiolepis*, and sawflies preferred experimental plants with long shoots whether relatively low or high in phenolics (Waring and Price, 1988). Among natural willow clones, total phenolics provided no explanation of sawfly densities (Boecklen et al., 1990). More detailed studies on within-plant variation showed that in the host plant, *Salix lasiolepis*, longer shoots had higher concentrations of specific phenolic glucosides, with tremulacin and salicortin the most concentrated glucosides present (Price et al., 1989). With phenolic glucosides as the major chemical defenses of many willow species and the major feeding cues for other herbivores (e.g., Lindroth et al., 1988; Rank, 1992; Soetens and Pasteels, 1994; Kolehmainen et al., 1995), we wondered if there were a simple relationship between their concentrations and female ovipositional choices. In our efforts to develop a mechanistic and factual theory on the popu-

lation dynamics of *Euura* sawflies, it was essential to understand the proximate mechanisms involved with host plant shoot selection.

A breakthrough in the chemical ecology of *Euura* species came with the demonstration that phenolic glucosides were central in host selection of the European shoot-galling sawfly *Euura amerinae* on *Salix pentandra* (Kolehmainen et al., 1994). As in other *Euura* species, females prefer to oviposit into long shoots on young plants (Roininen et al., 1993a, 1995). Females selected their host plant for oviposition based on phenolic glucoside content, especially the main constituent, 2′-*O*-acetylsalicortin, and within host plants this compound is probably involved in shoot selection (Kolehmainen et al., 1994). Therefore, we undertook comparable studies with the American shoot-galling sawfly, *Euura lasiolepis*.

We asked the following questions: (1) On willows not encountered by *E. lasiolepis* naturally, is oviposition stimulated by host species with phenolic glucosides the same as those in its natural host, *Salix lasiolepis?* (2) Which individual phenolic glucosides stimulate oviposition the most? In both cases our hypothesis was that *E. lasiolepis* females would respond most strongly to the most concentrated glucosides, tremulacin and salicortin, in their natural host plant. Because these two glucosides were similar in concentration and positively correlated (Price et al., 1989), we could make no prediction on their relative roles (mean for salicortin 15.69 mg/g dry wt, and for tremulacin 15.35 mg/g dry wt; $Y = 1.12x - 2.24$, $N = 12$, $r^2 = 0.75$, $P < 0.01$).

METHODS AND MATERIALS

Question 1: Multiple-Choice Test with Plant Species. The first test was conducted with six willow species in the family Salicaceae from around Joensuu, Finland, and *S. lasiolepis* from Flagstaff, Arizona, USA. The species were *Salix caprea*, *S. myrsinifolia*, *S. pentandra*, *S. phylicifolia*, *S. purpurea*, and *S. rosmarinifolia* from Finland and *S. lasiolepis* from the USA. The second test included the six Salicaceae listed above from Joensuu and *Populus tremula*, a common salicaceous species in Finland. *Euura lasiolepis* galls were collected in the field in the winter and stored in a freezer. Female sawflies emerged from galls that were raised to room temperature a few weeks before the start of the experiments. Each female was caged for 24 hr with two to three rapidly growing shoots of each plant species by repeating the experimental conditions described in Roininen and Tahvanainen (1989). The first test included 11 replicate cages, and the second test included 15 replicate cages.

The number of test scars made with the ovipositor and the number of eggs actually laid by sawfly females were recorded. Nonparametric Friedman two-way analysis of variance was employed to detect any differences among plant species in the number of oviposition scars (Siegel, 1956).

The phenolic glucoside contents of leaves and twigs of the Finnish Salicaceae have been described in a series of papers (Julkunen-Tiitto, 1985, 1986; Tahvanainen et al., 1985a,b; Meier et al., 1988; Roininen et al., 1993b; Kolehmainen et al., 1994, 1995). The natural host of *Euura lasiolepis* in Arizona, *Salix lasiolepis*, also has had its phenolic glucosides characterized by the same methods (Price et al., 1989). Hence, we have directly comparable data on the chemical content of the natural host of the sawfly, and the willows and the aspen in Finland that were used in the bioassay.

Question 2: No-Choice Tests with Phenolic Glucosides and Salicylalcohol. The method employed has been described in Kolehmainen et al. (1994). A piece of green filter paper (3 × 5 cm) was placed in each Petri dish (diameter 9 cm). One test liquid applied on the filter paper was exposed to a single female sawfly for 3 hr. For three 20-min periods, the female was observed and the number of oviposition attempts was recorded. Total observation time per female was 60 min. If females became highly stimulated to probe they would do so repeatedly, so the duration of probing was recorded when observed.

Phenolic glucosides tested were either pure products available commercially (arbutin, salicin, and salicylalcohol) or fractions extracted from willows of known composition (tremulacin and salicortin). The tremulacin fraction contained 90.4% tremulacin, 5.7% tremuloidin, 1% salicylalcohol, and a trace of other glucosides. The salicortin fraction contained at least 93.0% salicortin, 3.5% salicin, and less than 3.5% of unknown glucosides depending on extraction. Purification of fractions were as in Kolehmainen et al. (1994) and quantification as in Julkunen-Tiitto et al. (1996).

Concentrations of phenolic glucosides in test solutions were 1.5%. Tremulacin and salicortin fractions were also tested as 0.4% concentrations in order to detect any effects of concentration of the most common glucosides on oviposition behavior, although in the study of Kolehmainen et al. (1994), concentrations of individual phenolic glucosides of 0.45% and 4.5% caused no significant differences in responses of *E. amerinae* under the same experimental conditions as employed in this study. Both concentrations used in this study are in the natural range of concentrations found in nature.

All tested compounds except the tremulacin fraction were dissolved in distilled water. The tremulacin fraction, which is not fully soluble in water, was dissolved in 30% ethanol. Test solutions (100 μl) were applied to each piece of filter paper. For the tremulacin treatment, ethanol was evaporated from filter paper for 6–10 min after which 30 μl of water was added to the filter paper. Pieces of filter paper treated with the solvent (water for most treatments) served as control. Tremulacin controls and part of the salicortin replicates containing 30% ethanol were prepared following the procedure used for treatment preparation. There were no effects of ethanol in controls or in salicortin treatments (Mann-Whitney test for both cases: $P > 0.10$).

The number of replicates per test liquid ranged from 10 to 67 females, depending on the observed variation. If there were no effects of a test liquid with 10 females, no more tests were conducted, but the control liquid tests were run each time with test liquids. Nonparametric Kruskal-Wallis one-way analysis of variance was used to detect any differences between treatments (Siegel, 1956).

RESULTS

Phytochemical Content of Plants. Phenolic glucoside contents of *Salix lasiolepis* and the Finnish Salicaceae tested have been published previously and are given in Table 1. *Salix lasiolepis*, with its relatively high concentrations of salicortin and tremulacin, was most similar to *S. purpurea* and *S. rosmarinifolia*, but distinctly different from the other Finnish willow species in glucoside content.

Multiple Choice Tests with Plants. Ovipositing females strongly preferred their original host plant, *S. lasiolepis*, when it was offered with members of the Finnish Salicaceae (Table 2). When only Finnish Salicaceae were offered, ovipositing females indicated a significant preference for *S. rosmarinifolia* (Table 3). Other species also were scarred, and *S. caprea* even had a few eggs laid in it. However, none of the Finnish willows was comparable to *S. lasiolepis* in stimulating a strong ovipositional response (Table 2). Females of the Finnish sawfly *E. amerinae* made more than 20 oviposition scars on its primary host *S. pentandra* under similar experimental conditions (Kolehmainen et al., 1994), while *E. atra* females made over 150 scars on their host *S. rosmarinifolia* in their lifetime (Roininen et al., 1993b). However, it is interesting to note that in the present experiment one plant species with relatively high salicortin and tremulacin concentrations, *S. rosmarinifolia*, elicited a response by the sawfly, while another one with rather similar glucoside content, *S. purpurea*, did not (Tables 1 and 3).

No-Choice Tests with Phenolic Glucosides. The mean number of testings with the ovipositor per hour by female *E. lasiolepis* was significantly higher on the tremulacin fractions than on any of the other treatments and the control (Figure 1). Tremulacin at 1.5% caused significantly higher probing than tremulacin at 0.4% (Mann-Whitney test, $U = 142.5$, $N = 54$, $P = 0.001$). Probes on 1.5% tremulacin were at least 19 times more frequent than on other glucosides and salicylalcohol (Figure 1). Once stimulated to probe, the only glucoside to elicit continuing probing was tremulacin, with mean duration of a probe of 6.4 ± 1.4 sec (mean of 16 females $\pm$ 1 SE). Other glucosides stimulated only a few probes lasting less than 1 sec each.

These results coincide well with the phenolic glucoside content of the natural host of *E. lasiolepis* (Table 1). Although tremulacin and salicortin are similar

TABLE 1. MEAN PHENOLIC GLUCOSIDE CONTENT IN LEAVES OF HOST PLANT, *S. lasiolepis*, AND FINNISH SALICACEAE USED IN MULTIPLE-CHOICE EXPERIMENTS[a]

	Content (mg/g dry wt)							
Glucoside	*S. lasiolepis*	*S. caprea*	*S. myrsinifolia*	*S. pentandra*	*S. phylicifolia*	*S. purpurea*	*S. rosmarinifolia*	*P. tremula*
2′-*O*-acetylsalicin	1.0	0.08	0.23	0.42	0.06	0.40	0.84	0.064
Picein	0.9				0.08			
Salicin	1.6	0.38	3.55	0.45	0.06	2.00	13.4	0.58
Salicortin	15.7	0.34	28.28	0.26	0.05	53.7	67.6	5.47
Salidroside					0.18			0.072
Tremulacin	15.4					16.7	18.7	
Tremuloidin	1.8					0.6	2.8	
Triandrin		0.09			0.24			

[a]Standard errors of the mean may be obtained from original reports: Price et al. (1989) for *S. lasiolepis*, Meier et al. (1988) for *S. rosmarinifolia*, and Julkunen-Tiitto (1986) for the remainder except for *S. purpurea* (Julkunen-Tiitto, 1989).

TABLE 2. RESULTS OF MULTIPLE-CHOICE OVIPOSITION TESTS WITH *S. lasiolepis* FROM ARIZONA, USA, AND MEMBERS OF SALICACEAE FROM FINLAND

Plant species	Oviposition scars per female (mean ± SE)[a]	Females causing scars (%)	Eggs laid per female (mean ± SE)	Females laying eggs (%)
S. lasiolepis	7.7 ± 1.0	100	2.0 ± 0.5	81
S. rosmarinifolia	0.3 ± 0.2	18	0	0
S. caprea	1.0 ± 0.6	18	0.1 ± 0.1	9
S. phylicifolia	0	0	0	0
S. pentandra	0	0	0	0
S. purpurea	0.1 ± 0.1	9	0	0
S. myrsinifolia	0	0	0	0

[a]Friedman two-way ANOVA, $df = 6$, $\chi^2 = 53.05$, $P < 0.001$. Other columns are given as additional relevant data.

TABLE 3. RESULTS OF MULTIPLE-CHOICE OVIPOSITION TESTS WITH MEMBERS OF SALICACEAE IN FINLAND, WITH SPECIES LISTED IN ORDER OF PREFERENCE

Plant species	Oviposition scars per female (mean ± SE)[a]	Females causing scars (%)	Eggs laid per female (mean ± SE)	Females laying eggs (%)
S. rosmarinifolia	6.5 ± 3.9	60	0.3 ± 0.2	13
S. caprea	0.4 ± 0.2	27	0.1 ± 0.1	13
S. phylicifolia	0.3 ± 0.3	7	0	0
S. pentandra	0.3 ± 0.2	13	0	0
S. purpurea	0	0	0	0
S. myrsinifolia	0	0	0	0
P. tremula	0	0	0	0

[a]Friedman two-way ANOVA, $df = 6$, $\chi^2 = 11.34$, $P < 0.045$. Other columns are given as additional relevant data.

in their concentration in *S. lasiolepis*, clearly the tremulacin fractions are much more attractive to sawflies than the salicortin fractions.

DISCUSSION

The evidence presented leads to the conclusion that tremulacin in the host plant, *S. lasiolepis*, is a key oviposition stimulant for the stem-galling sawfly, *E. lasiolepis*. Tremulacin is a major constitutent of the phenolic glucosides in *S. lasiolepis*. At least one foreign plant with tremulacin, *S. rosmarinifolia*, also elicited a clear ovipositional response. Among the several tested phenolics, trem-

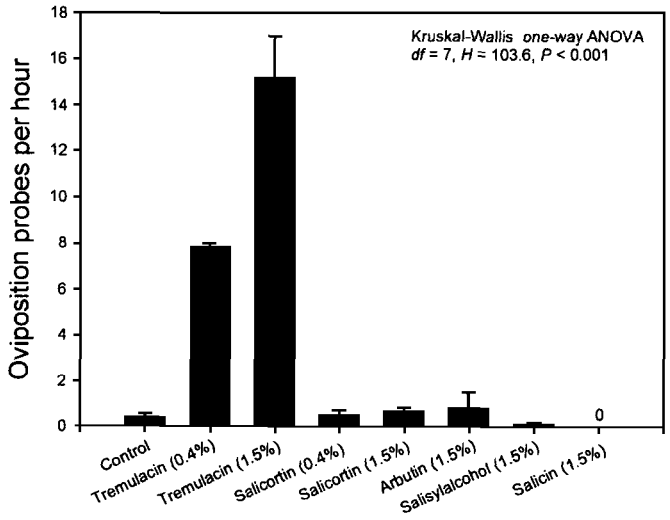

FIG. 1. Mean (± SE) number of tests by ovipositor per female *E. lasiolepis* in no-choice tests of the control (pure water) and individual phenolic glucosides or fractions.

ulacin was the only test substance that elicited a strong ovipositional response, and the only one to result in prolonged probing behavior by female sawflies.

Obviously, we have generated some questions that remain unanswered. If tremulacin is a key stimulus for oviposition, why did *S. purpurea*, which also has a high tremulacin content, elicit no reaction? Can high levels of salicortin, such as in *S. purpurea* and *S. rosmarinifolia*, or possibly other components, act as oviposition suppressants? Tests with combinations of salicortin and tremulacin at differing concentrations may yield some answers. The small positive reactions by sawflies in multiple choice tests to willows with no detected tremulacin also raise a question of the possible confusing effects of coexisting plant species in such tests. Preferably, no-choice tests would be run simultaneously on each willow species, but these provide no guarantee that clear-cut rejection of nonhost species is obtained. Two studies on sawfly oviposition that used no-choice testing of host plants indicated some positive stimulus by nonhost plants under such conditions (Roininen and Tahvanainen, 1989; Roininen et al., 1993b).

As in the similar study on *E. amerinae* (Kolehmainen et al., 1994), long shoots of the host plant that the sawfly utilizes for oviposition have higher levels of phenolic glucosides than short shoots (Price et al., 1989). In the case of *S. lasiolepis*, long shoots also had higher concentrations of tremulacin than short shoots. Female sawflies may be responding positively to an ovipositional stimulant that results in utilization of the longest shoots available on a scale a female

can assess, such as a single plant. This would account for the repeated observations that females show a graded response to shoot length, accepting shorter shoot lengths as mean module length declines (Craig et al., 1989). A corollary is that females respond positively to a wide range of a particular phenolic glucoside, which thereby enables an appropriate response to shoot length whether females are foraging among generally long, rapidly growing shoots, or less vigorous shoots. The flexibility of the ovipositional response in relation to heterogeneity in concentration of stimulants in the host plant is no doubt an important adaptive feature in the plant–herbivore interaction.

The multiple roles of phenolic glucosides have been noted repeatedly as stimulants for insect herbivore feeding or oviposition, as defensive chemicals for the plant, or sequestered as defenses for the herbivore (Pasteels et al., 1988; Rowell-Rahier, 1984; Smiley et al., 1985; Tahvananinen et al., 1985b). The compromising effects of secondary compounds in the Salicaceae certainly raise questions about their role as defensive chemicals. In the case the *E. lasiolepis*, we have noted the weak or absent selective pressure imposed by insect herbivores relative to those from abiotic factors (Craig et al., 1988).

In the Salicaceae and insect herbivore systems involving chrysomelids and sawflies, we are fortunate to find that the most conspicuous and well-studied secondary compounds, the phenolic glucosides, play central roles as oviposition stimulants, phagostimulants, plant defenses, and insect herbivore defenses (Eisner, 1970; Kearsley and Whitham, 1993; Soetens and Pasteels, 1994). Advances in the chemical ecology of these interacting taxa could be made readily by broadening their comparative scope and extending studies to the third trophic level of carnivores.

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TISSUE-SPECIFIC FATTY ACID COMPOSITION OF GLANDULAR TRICHOMES OF MITE-RESISTANT AND -SUSCEPTIBLE *Pelargonium xhortorum*

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Abstract—Anacardic acids, alkyl phenolic acids excreted by tall glandular trichomes of the garden geranium, *Pelargonium xhortorum*, confer small-pest resistance. Up to 90% of the trichome exudate from mite-resistant *P. xhortorum* inbreds consists of an unusual anacardic acid with an unsaturated omega-5 (ω^5) alkyl chain. As fatty acids are biochemical precursors to anacardic acids, we examined by GC the fatty acid composition of leaves, pedicels, petals, sepals, mature seeds, and glandular trichomes from pest-resistant and pest-susceptible *Pelargonium* inbred lines to determine the localization of ω^5-fatty acids within plant tissues. The fatty acid composition of lipid classes (galactolipids, phospholipids, and neutral lipids) extracted from glandular trichomes from mite-resistant pedicels were also examined. ω^5-Fatty acids (16 : 1Δ11 and 18 : 1Δ13) were found only in the glandular trichomes from pest-resistant geraniums (27.2% in trichomes of pedicels) and were localized predominantly in the phospho- and galactolipids (phosphatidylinositol, 25.9%; phosphatidylcholine, 18.2%; monogalactosyldiglyceride, 15.5%; and diglactosyldiglyceride, 14.0%).

Key Words—Fatty acids, geraniums, glandular trichomes, glycolipids, *Pelargonium xhortorum*, phospholipids, ω^5-unsaturated fatty acids.

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INTRODUCTION

The discovery of susceptibility to the two-spotted spider mite (*Tetranychus urticae* Koch) in the normally mite-resistant garden geranium (*Pelargonium xhortorum* Bailey) by R. Craig (Snetsinger et al., 1966) resulted in the identification of anacardic acids in the exudate of glandular trichomes (Gerhold et al., 1984; Walters et al., 1988). Anacardic acids provide protection to the plant by acting as a sticky trap (Walters et al., 1989b), as an insect toxicant (Gerhold, 1984), and possibly by decreased pest fecundity (Grazzini et al., 1991; Walters et al., 1990a). The major anacardic acids in the exudate of resistant inbreds are uniquely unsaturated at the omega-5 (ω^5) position of the alkyl side chain (Walters et al., 1988). Susceptible inbreds produce mostly saturated anacardic acids, and contain very low levels (if any) of ω^5-anacardic acids. Chemical and bioassay analysis of F_1, F_2, and backcross progenies between homozygous resistant and susceptible geranium genotypes confirmed the association between the presence of ω^5-anacardic acid and mite resistance, and that ω^5-desaturation is conditioned by a dominant allele at a single locus (Grazzini et al., 1997). Analysis of glandular trichomes of 16 South African *Pelargonium* species indicated that *P. inquinans*, the putative progenitor of the common garden geranium, possessed ω^5-anacardic acids with a composition similar to the resistant inbred (line 71-17-7) used in these studies (Grazzini et al., 1995).

Anacardic acids are produced from the elongation of fatty acid precursors (Hesk et al., 1992; Walters et al., 1990b) without subsequent desaturation. Thus, ω^5-anacardic acids characteristic of resistant *Pelargonium* phenotypes and ω^5-fatty acid precursors should occur in resistant plants. However, such ω^5-fatty acids comprise only about 1% of the total fatty acid content of resistant *P. xhortorum* leaves, whereas ω^5-anacardic acids comprise up to 90% of the total anacardic acids produced (Walters et al., 1989b). In this study, we examine the fatty acid composition of trichomes from leaves, pedicels (hypantheum), sepals, and a mature seeds of resistant and susceptible *Pelargonium* inbreds; from the tissues after the glandular trichomes had been removed; and from the galacto-, phospho-, and neutral lipids from glandular trichomes of resistant *Pelargonium* inbreds.

METHODS AND MATERIALS

Plant Material

Homozygous diploid *Pelargonium* inbreds used were those previously determined by Walters et al. (1990a) to be true-breeding for bioassay response (resistant or susceptible), chemical composition (saturated or ω^5-unsaturated anacardic acids), and tall glandular trichome density (high or low density). The

red-flowered resistant inbred 71-17-7 and pink-flowered susceptible inbred 71-10-1 were clonally propagated and maintained in an active growing and flowering state in 4-liter pots under routine greenhouse conditions. Supplemental lighting was provided during the months of October–May giving a minimum daily photoperiod of 16 hr.

Part I. Fatty Acid Composition of Plant Tissue

Tissues with and without trichomes from leaves, pedicels, and sepals of resistant and susceptible genotypes were examined. Flower petals and mature seeds were also analyzed. Fresh tissue samples were harvested and immediately placed on Dry Ice until sample preparation and extraction. Tissue specimens were matched for approximate maturity. Pedicels, sepals, and petals were harvested from inflorescences in which about one third to one half of the florets were open and the petals reflexed. Mature seeds were obtained from bulked self-pollinations of each genotype.

Sample Preparation. Fresh tissue specimens were divided into replicates and prepared with the granular Dry Ice method of Yerger et al. (1992) to compare glandular trichome preparations and trichome-free preparations. After vortexing, trichome-free tissue pieces were removed with forceps, rather than by sieving, so that lipid extraction could occur in the same centrifuge tube as that used for sample preparation. Tissue specimens were homogenized in cold chloroform (C)–methanol (M) (2 : 1; v/v) in a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada). Extracts were kept on Dry Ice. Mature, dry seeds were ground in cold C-M (2 : 1; v/v) in a chilled mortar. Extracted samples were centrifuged and 2 ml of the supernatant removed for further processing. An aliquot of extract was washed with acidified water (HCl, ca. pH 3) (Folch et al., 1957), the organic phase removed, the solvent evaporated under nitrogen at 40°C, and the fatty acids in the residue were derivatized with boron trifluoride–methanol (Christie, 1982) to yield fatty acid methyl esters (FAMEs). Hexane extracts of the FAMEs were subsequently analyzed. Butylated hydroxytoluene (BHT) was added (ca. 0.01%) to solutions to decrease oxidation.

Gas Chromatographic Analysis. GC-FID analysis was conducted on a Hewlettt Packard 5890 GC with a RTX-2330 capillary column (30 m × 0.25 mm ID, 0.20-mm phase thickness) and a 1-m guard column (Restek, Bellefonte, Pennsylvania) with helium (7 psi, 20 cm/sec linear velocity) as a carrier gas. Injector temperature was 200°C, and the detector temperature was 260°C. Manual direct (off-column) injection (1 μl injection volume) was used with a packed column injector fitted to accept capillary columns. Data recording and integration were performed by a Shimadzu Chromatopac 5A recorder (Shimadzu Scientific Instruments, Columbia, Maryland). In routine operating conditions, we utilized repetitive 1-μl injections and a triple-ramped temperature program of

50–150°C at 10°C/min, 150–190°C at 5°C/min, 190–250°C at 20°C/min, with 250°C maintained for 10 min.

FAME retention times were compared with standards (RM-3, Supelco, Bellefonte, Pennsylvania) supplemented with 16 : 1 ω^5 and 18 : 1 ω^5-FAME prepared by Dr. David Hesk (Yerger et al., 1992), and 17 : 0, 17 : 0 anteiso, and 17 : 0 iso FAMEs (Larodan Chemical, Uppsala, Sweden). Retention time for 16 : 3 FAME was determined by extracting and analyzing the fatty acids of fresh market spinach known to be a 16 : 3 plant (Jaworski, 1987). Mass spectroscopy (MS) analysis was conducted with an Hewlett Packard 5970 mass spectroscope. For additional verification, sample aliquots were hydrogenated over a platinum catalyst or derivatized with dimethyl disulfide (DMDS) (Hesk et al., 1991) and analyzed by GC-MS to determine double bond positions.

Part II. Fatty Acid Composition of Lipids of Glandular Trichomes

Glandular Trichome Preparation. Trichomes were harvested from pedicels as described in Yerger et al. (1992), with modifications by Hage (1995), as performed by Paul (1995). Only pedicels were used because of the high and near-exclusive abundance of tall glandular trichomes and ease of harvesting and preparation. In this procedure, no powdered Dry Ice was used to shear off trichomes as used in part I.

Lipid Extraction. The screened trichomes were homogenized in C-M (2 : 1; v/v) in an Ultra-Turrax homogenizer (Tekmar Company, Cincinnati, Ohio), and then sonicated for 60 min in a Branson 1200 Ultrasonic Cleaner (Branson Ultrasonics Corporation, Danbury, Connecticut). Following centrifugation, the extract was evaporated to dryness and the residue redissolved in 4 ml of 2 : 1 C-M and washed (Folch et al., 1957) as described for samples in part I.

Lipid Class Separation by Column Chromatography. For separation of lipid classes, we used the following modification of the method of Whitaker (1991). A glass chromatographic column (1.25 cm ID × 29.6 cm) was wet-packed with silica gel (Kieselgel 60, 70–230 mesh ASTM, EM Science, Darmstadt, Germany) in chloroform to a length of 7.5 cm. The column was washed with 50 ml of chloroform and an aliquot of the trichome extract (approximately 500 μl) was loaded on top of the column. The column was eluted first with 175 ml of chloroform–acetone (20 : 1; v/v) to collect neutral lipids, and then with 175 ml of acetone–methanol (20 : 1; v/v) to elute the glycolipid fraction. Polar lipids, primarily phospholipids, were eluted with 175 ml of methanol. The three fractions were individually evaporated to dryness in a rotary evaporator and each residue was redissolved in 1 ml of solvent (chloroform for the chloroform fraction, 2 : 1 chloroform–methanol for the acetone fraction, and 1 : 1 chloroform–methanol for the methanol fraction).

Isolation of Individual Lipids from Lipid Classes by Thin-Layer Chromatog-

raphy. Each of the three lipid fractions was further separated into specific lipid bands via thin-layer chromatography (Merck plates, 20 × 20 cm, 0.25 mm thickness Kieselgel 60 F_{254}) with a preconcentration zone (EM Science, Darmstadt, Germany). The neutral lipid fraction was separated by using a mobile phase of benzene–diethyl ether–ethanol–acetic acid (50 : 40 : 2 : 0.2, by vol) (Freeman and West, 1966), while the glycolipid and phospholipid fractions were separated in chloroform–methanol–water (65 : 25 : 4, v/v/v) (LePage, 1964). After the plates were dried, a glass was placed over the center of the plates and the separated lipid bands were visualized on the edges by staining with iodine. Bands also were identified by cochromatography with lipid standards. Lipid detecting sprays (α-naphthol, ninhydrin, and Phospray) (Supelco, Bellefonte, Pennsylvania) were also used. With the iodine-stained portion of each plate as a guide, we scraped unstained lipid bands into 20-ml vials and the lipids were dissolved with 2 : 1 C-M (for the neutral lipid and glycolipid bands) or 1 : 1 C-M (for the polar lipid bands). The vials were sonicated for 30 min, after which the silica gel was separated by centrifugation. The resulting supernatant was transferred to a 4-ml vial, evaporated to dryness, and methylated for GC analysis as described earlier.

RESULTS

GC Characterization of FAMEs. The FAME pairs 16 : 1Δ9(ω^7) and 16 : 1Δ11(ω^5) as well as 18 : 1Δ9(ω^9) and 18 : 1Δ13(ω^5) were resolved cleanly by GC. GC-MS was used to determine the molecular weights of FAMEs, to analyze the hydrogenated aliquots of the FAMEs, and to identify the DMDS adducts. The two tentatively identified ω^5-fatty acids gave DMDS derivatives of the FAMEs with GC retention times identical with standards and completely separate from the DMDS derivatives of 16 : 1 ω^7 and 18 : 1 ω^9 (4 and 9 sec, respectively). The DMDS of the FAMEs of standard 16 : 1 ω^5 and the tentatively identified 16 : 1 ω^5 from the extract gave identical mass spectra and prominent ions at m/z = 117 and 245, characteristic of a 16-carbon fatty acid methyl ester with an ω^5-double bond. The DMDS of FAMEs of standard 18 : 1 ω^5 and the tentatively identified 18 : 1 ω^5 gave identical spectra and strong ions at m/z = 117 and 273, characteristic of an 18-carbon fatty acid methyl ester with an ω^5-double bond. From these data, we unequivocally identified the two unique unsaturated fatty acids of the glandular trichomes as 16 : 1 ω^5 and 18 : 1 ω^5. The 16 : 3 fatty acid, characteristic of the "prokaryotic" plastid galactolipid desaturation pathway, was tentatively identified in *Pelargonium* extracts by reference to a standard prepared from fresh market spinach. We observed the presence of trace amounts of a monoenioic 17-carbon fatty acid methyl ester (m/z = 282), which we tentatively identified as 17 : 1 ω^6 as predicted by Hesk et al. (1992).

Following hydrogenation of trichome extracts, all compounds tentatively

identified as being unsaturated disappeared from the chromatogram, with a corresponding increase in area within the saturated FAME peak of the same carbon number. Anteiso 17:0- and 16:1Δ11(ω^5)-FAMEs did not resolve under routine GC operating conditions. A new capillary column in which the RTX-2330 phase had not been used separated these two FAMEs but would not do so after a few weeks, thus requiring GC-MS analysis. Prior to hydrogenation, 24.9% 16:1Δ11(ω^5)- and 10.5% 18:1Δ13(ω^5)-FAMEs were detected in a resistant trichome preparation. Following hydrogenation, neither ω^5-FAME was observed, but a small amount (5.0%) of 17:0 anteiso FAME was present, apparently obscured in the analysis of the native trichome preparation by the 16:1Δ11(ω^5). Similar amounts of 17:0 anteiso (5.3%) were detected in the susceptible extracts following hydrogenation, indicating that branched-chain fatty acid synthesis, which ultimately is derived from the amino acid leucine, simultaneously occurs in trichomes of both resistant and susceptible inbreds. No ω^5-fatty acids were observed in susceptible tissue, contrary to the report of traces of 16:1Δ11(ω^5)-unsaturated fatty acid (Walters et al., 1990b).

Fatty Acid Composition of Leaf, Flower, and Seed Tissue. ω^5-Fatty acids were observed in the resistant plant almost exclusively in the glandular trichomes, 27.2% in pedicel trichomes, but not in any susceptible tissue (Tables 1 and 2). ω^5-Fatty acids were not observed in resistant tissues from which trichomes had been removed, except for a trace in pedicel tissue, which was probably contaminated with trichomes. Similarly, 17:1Δ11(ω^6) was detected only in resistant tissues, and was concentrated in glandular trichome preparations, indicating glandular trichome-localized biosynthesis of both of these fatty acids. Otherwise, the fatty acid composition of leaf, pedicel and sepal tissue fractions of resistant and susceptible inbreds is very similar, with 16:0, 18:2 and 18:3 as the dominant fatty acids. The 16:3[Δ7, 10, 13 ($\omega^{3,6,9}$)] fatty acid characteristic of the prokaryotic lipid desaturation mechanism (Jaworski, 1987) was observed in low levels in all resistant and most susceptible tissues analyzed. Small amounts of 16:1 ω^7, 17:0 anteiso and 18:0 iso were observed.

Fatty Acid Composition of Petals. As petals do not generally bear glandular trichomes, only whole petal preparations were analyzed (Tables 1 and 2). Resistant petal tissue contained relatively high amounts of saturated fatty acids (16:0, 18:0, 20:0, 24:0) and only possessed an unsaturation index (UI) of 61.1 (Mumma et al., 1971). Susceptible petals were much more unsaturated (UI = 112.3). Trace amounts of ω^5-fatty acids in resistant petal preparations may reflect either the small number of tall glandular trichomes present on the sepals or contamination. Petals from resistant and susceptible plants possessed significant amounts of 22:0 and 24:0 that are likely incorporated in the cuticular waxes.

Fatty Acid Composition and Anacardic Acid Composition of Pelargonium *Seeds.* No ω^5-fatty acids or -anacardic acids were observed in seeds of either

TABLE 1. MEAN PERCENTAGE OF FATTY ACID METHYL ESTER COMPOSITION OF GLANDULAR TRICHOMES (T) AND TRICHOME-FREE TISSUE (TF), LEAVES (LF), PEDICELS (PED), SEPALS (SEP), PETALS, AND SEEDS FROM MITE-RESISTANT *Pelargonium* INBRED 71-17-7

	Relative percent of fatty acids[a]							
Fatty acid	T-LF	TF-LF	T-PED	TF-PED	T-SEP	TF-SEP	Petals	Seeds
16:0	24.7	24.1	23.2	31.6	17.6	29.7	36.6	31.9
16:1 ω^7	2.0	2.6	1.5	1.2	0	1.6	0.3	0.1
17:0 anteiso	0.0	0.1	1.3	0	0	0	0	0
16:1 ω^5	**7.6**	**0**	**18.4**	**0.1**	**11.7**	**0**	**0.2**	**0**
17:0	0.7	0.5	1.1	0.7	0.5	0.3	0.6	0.2
18:0 iso	0.8	0.5	0.8	0.1	0.4	0.3	0.1	0
17:1 ω^6	**0.4**	**0**	**2.1**	**0.2**	**0.8**	**0.1**	**0.1**	**0**
18:0	9.2	8.0	7.8	11.3	11.9	2.7	23.4	2.7
16:3	0.4	1.0	0.4	0.2	0.1	0.4	0.1	0
18:1 ω^9	5.4	3.6	6.1	4.4	6.6	3.4	3.3	15.7
18:1 ω^5	**2.7**	**0**	**8.8**	**0.1**	**6.9**	**0**	**0.1**	**0**
18:2	27.0	18.2	23.6	32.3	26.9	26.7	20.4	44.8
20:0	0.5	0.2	0.9	0.7	1.2	0.3	1.0	0.4
18:3	26.8	40.6	7.7	18.9	9.3	28.8	5.6	3.5
22:0	0.5	0.2	0.8	0.2	7.1	6.3	3.6	0.3
24:0	0	0.3	0.8	0.2	0	0	4.8	0

[a]Average of (usually) two replicates; average SE ≤ 1.37%.

genotype. Seeds from both genotypes had similar fatty acid compositions (Tables 1 and 2).

Fatty Acids of Lipid Classes of Pedicel Trichomes. In trichomes prepared according to the procedure described in part II, ω^5-fatty acids constituted 10.3% of the fatty acids of the trichome extract (Table 3), compared to 27.2% in part I trichome preparation (Table 1), reflecting the more pure trichome preparation when Dry Ice was used. The neutral lipid fraction contained a small percentage of ω^5-fatty acids (3.4%). The fatty acids 18:0 and 16:0 accounted for over 75% of the fatty acids in the neutral lipid fraction (52.2% and 25.5%, respectively). The glycolipid and polar lipid (phospholipid) fractions contained 18.1% and 19.1% ω^5-fatty acids, respectively. The fact that both glycolipid and polar lipid fractions were high in 18:3 and 18:2 indicates that oxidation was not a significant problem in the isolation procedure. Even though the relative composition was low, the neutral lipid fraction contained ca. twice as much ω^5-fatty acids as the glycolipid fraction and the phospholipid fraction had approximately three times as much as the glycolipid fraction.

ω^5-Fatty Acids in TLC-Separated Bands of General Lipid Classes. Most of the ω^5-fatty acid (and other unsaturated fatty acids) in the neutral lipid frac-

TABLE 2. MEAN RELATIVE PERCENT FATTY ACID METHYL ESTER COMPOSITION OF GLANDULAR TRICHOMES (T), TRICHOME-FREE TISSUE (TF), LEAVES (LF), PEDICELS (PED), SEPALS (SEP), PETALS, AND SEEDS FROM MITE-SUSCEPTIBLE *Pelargonium* INBRED 71-10-1

	Relative percent fatty acids[a]							
Fatty acid	T-LF	TF-LF	T-PED	TF-PED	T-SEP	TF-SEP	Petals	Seeds
16:0	21.7	21.6	24.9	34.0	29.0	20.9	34.1	28.6
16:1 a7	0.9	2.8	1.3	0.5	1.4	1.1	0.1	0.1
17:0 anteiso	0	0.2	3.3	0.1	1.2	0	0	0
16:1 ω^5	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
17:0	1.6	0.5	2.0	0.8	2.2	0.3	0.2	0.1
18:0 iso	1.5	0.4	1.2	0.1	1.0	0.2	0	0
17:1 ω^6	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
16:3	0	1.7	0.7	0.3	0	0.3	0.2	0
18:0	18.7	8.8	8.4	16.2	6.1	4.7	3.8	3.5
18:1 ω^9	8.3	6.1	7.0	3.5	17.1	4.1	5.1	16.5
18:1 ω^5	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
18:2	28.2	14.7	36.3	22.1	25.7	26.5	33.6	46.5
20:0	1.2	0.3	0.6	0.6	3.4	0.2	0.9	0.5
18:3	15.0	43.0	12.4	21.9	9.8	39.4	13.3	3.7
22:0	1.1	0.1	1.0	0.2	3.2	0.3	4.1	0.4
24:0	0	0.1	1.1	0.2	0	0.3	3.8	0

[a]Average of (usually) two replicates; average SE ≤ 1.75%.

tion occurred in band 5, the highest migrating band on the plate. This band (R_f = 0.78) cochromatographed with triglycerides (TG) and contained a total of 10.6% ω^5-fatty acids. Interestingly, this was the only band in the neutral lipid fraction that was not dominated by 16:0 and 18:0. A somewhat smaller amount of ω^5-fatty acid was observed in the other neutral lipid bands, but lipid carryover between adjacent bands could have occurred (especially bands 4 and 5) because bands were not clearly differentiated. The glycolipid fraction separated into six bands on TLC, although only two of the bands had significant quantities of ω^5-fatty acids (Table 4). Band 2 (R_f = 0.45) representing digalactosyl diglyceride (DGDG), contained 14% ω^5-fatty acid. The most abundant component of band 2 was 18:3 (33.1%). Band 5 (R_f = 0.71), monogalactosyl diglyceride (MGDG) was composed of 15.5% ω^5 fatty acids. The most prevalent fatty acid in this band was 18:3 (32.0%). The presence of high levels of 18:3 in the DGDG and MGDG bands demonstrates good recovery from the TCL plate with a lack of substantial oxidation; moreover, 18:3 is reported to be the major fatty acid in plant galactolipids (Joyard and Douce, 1987). Of the four remaining faintly staining bands in the glycolipid fraction, only band 1 (R_f = 0.32) contained ω^2-

TABLE 3. MEAN PERCENTAGES FATTY ACID METHYL ESTERS OF GLANDULAR TRICHOME LIPID EXTRACT OF MITE-RESISTANT *Pelargonium* x *hortorum* AND NEUTRAL LIPID, GLYCOLIPID, AND POLAR LIPID FRACTIONS

	Relative percent fatty acid[a]			
Fatty Acid	Trichome extract	Neutral lipid[b]	Glycolipid[c]	Polar lipid[d]
16:0	21.4	25.5	12.8	13.6
16:1 ω^7	1.7	0.5	1.7	3.3
16:0 ω^5	**6.1**	**1.8**	**10.4**	**11.3**
18:0	31.9	52.2	6.5	3.4
18:1 ω^9	3.0	2.8	2.4	3.7
18:1 ω^5	**4.2**	**1.6**	**7.7**	**7.8**
18:2	22.2	9.0	22.8	44.8
18:3	9.7	6.6	35.9	12.2

[a]Average of two replicates; average SE ≤ 1.06%.
[b]Eluted from the silica gel column with 20:1 (v/v) chloroform-acetone.
[c]Eluted from the silica gel column with 20:1 (v/v) acetone–methanol.
[d]Eluted from the silica gel column with methanol.

fatty acids (6.5%). It did not give a positive reaction with the α-naphthol spray, although it contained over 60% 18:0 and 16:0. None of the four remaining bands in the glycolipid fraction was identified.

Thin-layer chromatography separated the polar lipid (phospholipid) fraction into five bands based on iodine staining. Band 1 (R_f = 0.125) contained a small amount of ω^5-fatty acids, and probably was a lysophosphatide. Bands 2–5 were high in ω^5-fatty acids. Band 2 (R_f = 0.21), phosphatidylinositol (PI), contained 25.9% ω^5-fatty acids. Band 3 (R_f = 0.28), phosphatidylcholine (PC), contained 18.2% ω^5-fatty acid. This band cochromatographed with phosphatidylcholine (PC) standard and gave a positive Phospray reaction. However, subsequent TLC in a different solvent system (chloroform–acetone–methanol–acetic acid–water 100:40:20:20:10 by vol) (Rouser et al., 1966) separated this band into two bands, one being PC while the other appeared to be phosphatidic acid (PA), possibly as a result of phospholipid degradation. Band 4 (R_f = 0.48), phosphatidylethanolamine (PE), was composed of 8.4% ω^5 fatty acids. Band 5 (R_f = 0.89) contained 16% ω^5-fatty acids, but it migrated more than any polar lipid should be in 65:25:4 solvent system. It is not a pospholipid, but could be a degradation product of a phospholipid such as PC, as it is high in 18:2, as well as ω^5-fatty acids and the fatty acid content resembles most closely that of PC.

Cahoon and Ohlrogge (1994) determined that 18:2 was the most prevalent fatty acid in phospholipids of developing coriander and carrot endosperm and that PI had the lowest percent of 18:2 and the highest percent of 18:1 ω^{12} (a

TABLE 4. MEAN RELATIVE PERCENT FATTY ACID METHYL ESTERS OF TLC-SEPARATED BANDS OF THE GENERAL LIPID CLASSES FROM TOTAL GLANDULAR TRICHOME LIPID EXTRACT OF RESISTANT *Pelargonium* x *hortorum* INBREDS

Lipid fraction[a]	TLC band[b]	R_f	Relative percent fatty acid[c]							
			16:0	16:1 ω^7	**16:1 ω^5**	18:0	18:1 ω^9	**18:1 ω^5**	18:2	18:3
Neutral[d]										
	1, MG	0.24	30.3	0.1	**0.2**	67.5	0.6	**0.2**	0.7	0.5
	2	0.41	36.7	0.7	**1.6**	40.3	8.5	**2.4**	3.7	6.2
	3	0.57	28.5	1.3	**3.9**	38.4	6.6	**2.8**	11.8	6.8
	4, DG	0.71	27.9	0.1	**0.5**	68.1	1.0	**0.4**	1.4	0.7
	5, TG	0.78	20.2	2.2	**5.7**	9.8	10.1	**4.9**	25.9	21.5
Glycolipid[e,f]										
	1	0.32	43.7	2.9	**3.1**	20.6	7.8	**3.4**	11.1	7.5
	2, DGDG	0.45	18.7	1.4	**8.5**	12.8	4.8	**5.5**	15.3	33.1
	3, MGDG	0.71	16.1	0.8	**9.2**	13.7	3.4	**6.3**	18.6	32.0
Polar[e,g]										
	2, PI	0.21	21.3	4.8	**14.5**	8.6	3.2	**11.4**	27.2	9.3
	3, PC	0.28	13.9	3.2	**11.0**	3.9	4.0	**7.2**	45.6	11.2
	4, PE	0.48	25.6	1.3	**4.7**	8.0	4.5	**3.7**	42.7	9.7
	5	0.89	17.2	3.1	**8.1**	7.1	4.8	**7.9**	40.3	11.5

[a]Neutral, glyco- and polar lipid fractions were obtained by sliica gel column separation of the total trichome lipid extract.
[b]MG = monoglyceride, DG = diglyceride, TG = triglyceride, DGDG = digalactosyldiglyceride, MGDG = monogalactosyldiglyceride, PI = phosphatidylinositol, PC = phosphatidylcholine, PE = phosphatidylethanolamine.
[c]Average of two replications.
[d]TLC solvent system was benzene–diethyl ether–ethanol–acetic acid (50:40:2.0:0.2, v/v.)
[e]TLC solvent system was chloroform–methanol–water (65:25:4, v/v).
[f]Bands 3, 4, and 6 contained negligible amounts of fatty acids (based on GC peak areas) and are not reported.
[g]Band 1 contained negligible amounts of fatty acids (based on GC peaks areas) and is not reported.

unique fatty acid found in the seed oil of most Umbelliferae, Araliaceae, and Gerryaceae species) of all the phospholipids. We observed similar results with the glandular trichome polar lipid fraction; PI had the lowest percent of 18 : 2 (27.2%) and the highest percent of ω^5-fatty acid (25.9%).

Based on GC peak area data, PC had the greatest amount of ω^5-fatty acid, followed by PI, and then PE. Cahoon and Ohlrogge (1994) reported that, of the phospholipids in developing coriander and carrot endosperm, petroselinic acid was the lowest (in percent composition) in PE, which is what we observed regarding ω^5-fatty acid. It must be noted that other phospholipids undoubtedly are present in our preparation (especially phosphatidylglycerol) in quantities below our detection levels.

DISCUSSION

The ω^5-fatty acids were localized only in the tall glandular trichomes of small pest-resistant *Pelargonium* inbreds. No ω^5-fatty acids were detected in susceptible leaf tissue, as previously reported by Walters et al. (1990b). No ω^5-fatty acids were found in tissues from which tall glandular trichomes had been removed, nor were any identified in storage tissues such as seeds. In all tissues examined, 16 : 0, 18 : 0, 16 : 1ω^7, 18 : 1, 18 : 3 and frequently a hexadecatrieneoic acid (16 : 3) were present. These observations indicate that fatty acid synthesis, elongation, Δ9 desaturation, Δ12 desaturation, and Δ15 desaturation all occur in *Pelargonium*, and at approximately the same levels in both resistant and susceptible genotypes.

Fatty acid synthesis typically initiates with an acetyl-CoA starter molecule (Stumpf, 1987). The acetyl-CoA carboxylase enzyme that binds the acetyl-CoA primer is not entirely specific for acetyl-CoA. Three-carbon CoA molecules can be used as primers (Walters and Steffens, 1990) giving rise to odd-carbon-number fatty acids, or anteiso or isobranched-chain fatty acids. The presence of odd-carbon-number and branched chain anacardic acids indicated that there must be odd-carbon-number and branched-chain fatty acid precursors to these minor anacardic acids, and the present report confirms this. Hesk et al. (1992) also reported the presence of a 23 : 1 ω^6-anacardic acid in resistant inbreds, and suggested that a 17 : 1Δ11(ω^6)-fatty acid precursor must also exist in the glandular trichomes. We observed this 17 : 1Δ11(ω^6)-fatty acid in all tissues in which there were ω^5-fatty acids.

Because the position of the double bond is consistent (in the ω^5 position) in the two major anacardic acids (C_{22} and C_{24}) of resistant inbreds, it has been convenient to refer to these anacardic acids as ω^5-anacardic acids, but this convenience is misleading. The presence of the 17 : 1Δ11(ω^6) fatty acid is an indication that the desaturase counts in from the carboxyl end of the fatty acid: a Δ-desat-

urase, not an ω-desaturase. There could be more than one desaturase involved, but genetic studies (Grazzini et al., 1997; Schultz et al., 1996) indicate only one gene is responsible for mite susceptibility; thus we expect the Δ desaturase acts on primarily one fatty acid, which then undergoes chain elongation to form other fatty acids. The logically expected desaturase was a Δ11 16 : 0 desaturase; but subsequent work has shown the enzyme to be a Δ9 14 : 0 desaturase that can act to a limited degree on 15 : 0 fatty acids (Schultz et al., 1996). The 14 : 1Δ9(ω^5)-fatty acid is then chain elongated to 16 : 1Δ11 and 18 : 1Δ13 fatty acids.

In this report, we demonstrate that 16 : 1(ω^5), 17 : 1(ω^6), and 18 : 1(ω^5) fatty acids represent 10.7–29.2% of the total fatty acids of the glandular trichomes isolated by shearing action of Dry Ice. This relatively high concentration suggests a pool of ω^5-fatty acids is available for biosynthesis of anacardic acids. We have shown previously that excreted anacardic acids are removed from the glandular trichome head when rinsed with water (Walters et al., 1989a), as it would be with rain, producing susceptible plants. Moreover, increasing temperatures reduce the viscosity of the exudate, such that it flows off the glandular heads to the stem support cells, resulting in the loss of small arthropod resistance (Walters et al., 1991). This meltdown occurs at temperatures as low as 25.5°C. In both of the above cases, anacardic acids need to be biosynthesized and excreted in order to restore resistance. A pool of ω^5-fatty acids ensures that anacardic acid biosynthesis could proceed when needed.

The ω^5-fatty acids were observed in all three general lipid classes (neutral, glyco- and polar lipid) with the majority occurring in the polar lipids and highest concentrations in the phospholipids. That ω^5-fatty acids were distributed in all three general lipid classes, as well as many of the specific lipids within each class, implies that these unique fatty acids are not located within a single specific lipid pool inside a trichome. The high concentrations of ω^5-fatty acids found in the phospho- and glycolipids are interesting, since these lipids are found throughout the membranes of the secreting single trichome cell, suggesting that the permeability of these membranes containing high concentrations of ω^5-fatty acids may differ from other plant cells.

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THE PLANT GROWTH INHIBITOR NAGILACTONE DOES NOT WORK DIRECTLY IN A STABILIZED *Podocarpus nagi* FOREST

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Abstract—Nagilactones isolated from *Podocarpus nagi* are known for poisonous physiological activities to organisms. To clarify the allelopathic potential of nagilactone to wild plants, the seasonal changes in nagilactone contents in throughfall, leaf litter, and soil were monitored monthly in a podocarp forest at Mt. Mikasa, Nara City, central Japan. The average annual nagilactone concentrations in throughfall, leaf litter, and surface soil at 0–10 cm depth were 4.7×10^{-12} g/ml, 3.8×10^{-4} g/g and 3.1×10^{-7} g/g, respectively. The nagilactone flux from canopy leaves to the forest floor via throughfall was 5.2×10^{-2} g/ha/yr, which was far smaller than that via leaf litterfall: 765.3 g/ha/year. The disappearance of nagilactones from leaf litter and the nagilactone accumulation in the Ao layer and mineral soil were also studied. Nagilactone in leaf litter rapidly disappeared and its relative disappearance rate was 5.8/yr. Nagilactone concentration in mineral soil also decreased rapidly with an increase in soil depth and could not be detected in soil at 40–100 cm depth. The total mass of nagilactones was 177.3 g/ha in the Ao layer and 105.0 g/ha in mineral soil. These quantities in nagilactone dynamics give a very rapid turnover time of 0.37 year (4.4 months) in a soil nagilactone pool, implying the possibility of the reabsorption of nagilactone by *P. nagi*, while the allelopathic effects of nagilactone to other plants in the podocarp forest are questionable.

Key Words—*Podocarpus nagi*, nagilactone, allelopathy, throughfall, leaching, leaf litter decomposition, litter bag, soil nagilactone content.

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INTRODUCTION

Nagilactone isolated from *Podocarpus nagi* (Thumb.) Zoll. et Moritz (Ohwi, 1965) by Hayashi et al. (1968, 1972) has six structural isomers (A, B, C, D, E, and F) and its physiological activities as an allelochemic are well known (Hayashi and Sakan, 1974; Suganuma, 1975). In a course of studies for clarifying the nagilactone dynamics in a *P. nagi* dominant forest, we considered the nagilactone as an antiherbivory substance and monitored seasonal change of nagilactone contents in leaves (Ohmae et al., 1996). In the present study, we investigate whether nagilactone acts as a plant growth inhibitor in natural forest conditions (Hayashi and Sakan, 1974).

If allelochemicals could directly work against the growth of other plants in situ, they might be either leached from living plants into throughfall (del Moral and Muller, 1969; Lee and Monsi, 1963; Molina et al., 1991) or released from decaying leaf litter (Chou and Patrick, 1976; Gant and Clebsch, 1975; Neilson and Richards, 1989). Allelochemicals dissolved in throughfall are water-soluble and waterborne (McPherson and Muller, 1969) and thus are directly and rapidly available for root absorbtion by plants. In the process of leaf litter decay, water-soluble allelochemicals are easily leached out (Rice, 1984), while water-insoluble allelochemicals in litter exert a strong influence over the decomposition process (Gallardo and Merino, 1993). Allelochemicals transported through different pathways will finally permeate into soil and then are either decomposed by soil microorganisms or taken up by plants. In addition to the release rate of allelochemicals from leaf litter, seasonal changes in allelochemical content in throughfall and soil should be quantified in order to understand the dynamic processes of allelochemicals more completely. However, only a few reports have studied quantitatively the dynamic features of the chemicals concerned (Heisey, 1990). In the present study, nagilactone concentrations were determined for throughful, leaf litter, and soil. Nagilactones A, B, and C received special attention in the present study.

METHODS AND MATERIALS

Study Forest. This research was conducted in the *Podocarpus nagi* forest on the western slope of a low hill, Mt. Mikasa. The ecological characteristics of the forest were described previously by Ohmae et al. (1996). A research plot of 30×30 m was established in the *P. nagi* forest in May 1991. Details of the plot are abbreviated here, since they were given fully in an earlier study (Ohmae et al., 1996).

The sampling of throughfall, leaf litter, and surface soil at 0–10 cm depth was conducted each month within the research plot in 1991 and 1992. Samples

of the Ao horizon layer and mineral soil were collected by changing sampling soil depth in July 1991. A litter bag experiment was also conducted in the plot to help understand the disappearance of nagilactone from leaf litter.

Throughfall Samples. An incident precipitation concept proposed by Leonard (1966) was applied to this study and was defined as rainfall that was recorded at the Nara Meterological Station (104 m alt.) about 2.5 km northwest of the research plot at Mt. Mikasa. A throughfall collector was made from a polyethylene bottle with a capacity of 10 liters and a 50-cm-diameter funnel. The bottle and funnel were glued together. The mouth of the funnel was 60 cm in height to prevent soil from splashig into the collector. Ten sites were selected randomly in the research plot and a throughfall collector was installed at each site. Throughfall was collected from all throughfall collectors once or twice per month or after heavy rain between August 1991 and August 1992. Collected throughfall was taken to the laboratory, filtered by suction to remove grit and organic debris, measured in volume, and concentrated with a rotary evaporator until its volume was reduced to about 200 ml. The concentrated throughfall was successively partitioned with dichloromethane and ethyl acetate. Crude solids for HPLC analysis of nagilactone were prepared by the same methods already described by Ohmae et al. (1996).

Leaf Litter Samples. Wang (1993) established 16 litter traps in the research plot in Mt. Mikasa to estimate the seasonal changes in litterfall. Leaf litter samples for the quantification of nagilactone content were collected with his litter traps from January 1992 to December 1992. A litter trap was 60 cm in diameter and stood 1.0 m above the ground. Podocarp leaf litter was collected once a month from all litter traps, taken to the laboratory, air-dried, and weighed. Crude solids for HPLC analysis were prepared by methods described previously (Ohmae et al., 1996).

Litter Bag Experiment. Fresh podocarp leaf litter for the litter bag experiment was sampled by establishing temporal litter traps in a defoliation season between June and July 1991 in Mt. Mikasa. Samples were taken to the laboratory and oven-dried. Each litter bag (15 × 15 cm) was made of nylon with a 1-mm opening and contained 7.0 g of podocarp leaf litter. Sixty litter bags were prepared and laid on the forest floor around the center of the research plot in July 1991. Five litter bags were collected randomly every month and returned to the laboratory. Decaying leaf litter was washed gently with 100% methanol to remove grit and organic debris, air-dried, and weighed. The methanol used for washing the litter was filtered and later used as the solvent for the crushed leaf litter. The crude solids from the litter were prepared for HPLC analysis (Ohmae et al., 1996).

The decomposition rate and half-life of the podocarp leaf litter were calculated by a mathematical model consisting of an exponential time function (Olson, 1963). The disappearance rate of nagilactones from leaf litter also was calculated by using the same model for leaf litter decomposition with respect to time.

Soil Samples. Following the conventional concept in soil science, soil was divided into two compartments, the Ao horizon layer and mineral soil (sensu soil). The Ao layer is the accumulation of litter on the forest floor and was sampled in seven quadrants (1 × 1 m) randomly located in the study plot during July 1991. A soil profile pit was dug to a depth of 1.0 m in July 1991. Mineral soil samples were collected at 10-cm intervals in depth by using a cylindrical core of 100 cm^3 volume. In addition, surface mineral soil at a depth of 0–10 cm was sampled once a month from September 1991 to September 1992. The amount of the monthly soil sample was in a range of 200–300 g dry weight.

Collected Ao layer samples were brought to the laboratory, dried in a ventilated air oven at 80°C for one week, weighed, and stored in pure methanol in glass bottles. Soil samples for chemical analysis also were taken to the laboratory, sieved, and stored in pure methanol in glass bottles. Crude solids for HPLC analysis were prepared by using methods described by Ohmae et al. (1996). Each soil sample was dried and weighed after suction filtration. Soil samples for the determination of the soil bulk density and the field capacity, as a measure of potential water content in soil, were handled following conventional laboratory methods in soil science (Kyuma et al., 1984). The decrease in rate of soil nagilactone content with respect to soil depth was approximated with a mathematical model consisting of an exponential function of soil depth (Ogawa et al., 1961).

Preparation of Samples for HPLC Analysis. HPLC equipment and conditions for the measurement of nagilactone content in all crude solids were similar to the methods described previously (Ohmae et al., 1996), although a HPLC column, Shiseido Capcell Pak C18 S-5 arg 6.0 × 250 mm was used in the present study.

RESULTS

Nagilactones in Throughfall. To compare throughfall with incident precipitation in the same unit, the unit of throughfall was changed from milliliters to millimeters for the rainfall unit. During the sampling period of throughfall, incident precipitation varied from 236 mm (October 1991) to 36 mm (February 1992). The quantities of incident precipitation and throughfall during the sampling period in 1991 and 1992 are 1507 mm/yr and 1236 mm/yr, respectively. Throughfall was closely related with incident precipitation and was expressed by a linear regression equation, $TH = 0.82P - 1.28$ ($r^2 = 0.97$), where TH is throughfall in millimeters and P is incident precipitation in millimeters. The interception loss at $P \ll 0$ is 1.28 mm, while its proportion to P at $P \gg 0$ is 17.9%. These figures are in a range of interception losses reported in forest hydrological records in Japan (Nakano, 1976). The regression equation between incident precipitation and throughfall was used to calculate the transfer of nagilactones from the leaf canopy to the forest floor by precipitation.

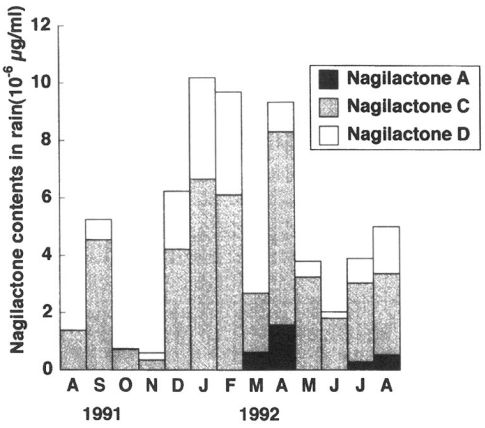

FIG. 1. Seasonal changes in nagilactone content in throughfall. Nagilactone content in each sample was divided into each sampling month. The annual average of nagilactone content in throughfall was 4.4×10^{-12} g/ml.

Collections of throughfall samples were made 18 times. The volume of throughfall and the nagilactone content in throughfall were measured for each sample. To understand the seasonal changes in nagilactone content, the quantities of nagilactone in each sample were divided into each collection month (Figure 1). Nagilactones C and D were leached out every month, but nagilactone A was leached out in only four months of the 13 observation months. Nagilactone C was highest in concentration among the three nagilactones throughout the sampling period. The total of the three nagilactones varied between 0.6×10^{-12} g/ml and 10.2×10^{-12} g/ml. Mean annual nagilactone content is 4.7×10^{-12} g/ml or 1.2×10^{-11} M in terms of the largest molecular weight of nagilactone C. This nagilactone content in throughfall is much smaller than the nagilactone concentration of 7×10^{-5} M for 50% growth inhibition of *Avena* coleoptiles (Hayashi and Sakan, 1974).

Nagilactone content had a slight positive correlation with the duration in sampling days and fine (clear) days during the sampling period, and a slight negative correlation with various rain indices. These results suggest that there is the possibility of leaching of nagilactones from the surface of leaves during fine days, but the nagilactone concentration in throughfall suggests that the quantity leached is very small. Thus, nagilactones are retained in living podocarp leaves and are released very little by throughfall to the forest floor.

Nagilactone Flux by Throughfall. Since rainfall changes from year to year, so does throughfall. To eliminate data fluctuation specific to the observation year, we estimated the mean annual throughfall from the mean annual rainfall at the

Nara Meteorological Station by using the linear relationship between throughfall and incident precipitation. According to climate data recorded from 1961 to 1990 at the meteorological station, mean annual incident precipitation was 1354.6 mm/yr. The corresponding calculated annual throughfall in the study plot is 1109.5 mm/yr. By multiplying the calculated annual throughfall by the mean annual concentration in throughfall (4.7×10^{-12} g/ml), the total mass of transported nagilactones was estimated as 0.052 g/ha/yr. The nagilactone flux thus estimated is unexpectedly smaller than the nagilactone accumulation in living mature leaves (3.9 kg/ha) (Ohmae et al., 1996).

Nagilactones in Leaf Litterfall. Figure 2a shows the seasonal changes in nagilactone content in leaf litter. Higher nagilactone concentrations in February, August, and October resulted from some unusual green leaves in leaf litterfall. Excluding these higher concentrations, the majority of the concentrations are in a range of less than 400 μg/g on a dry leaf weight basis. Nagilactone C was detected in all samples and had the highest concentration among the three isomers throughout the sampling period. The predominance of nagilactone C was similar to the nagilactone content observed in living mature leaves (Ohmae et al., 1996). A minor difference was observed between living and dead leaves with respect to the proportions of the three nagilactones. Nagilactones A, C, and D were detected in living mature leaves in six of 13 sampling months, while three isomers were detected from leaf litter in three of 12 months.

Nagilactone Flux by Leaf Litterfall. Nagilactones transported from the canopy to the forest floor through leaf litter were estimated by multiplying the mass of monthly podocarp leaf litterfall (Wang, 1993) with its corresponding

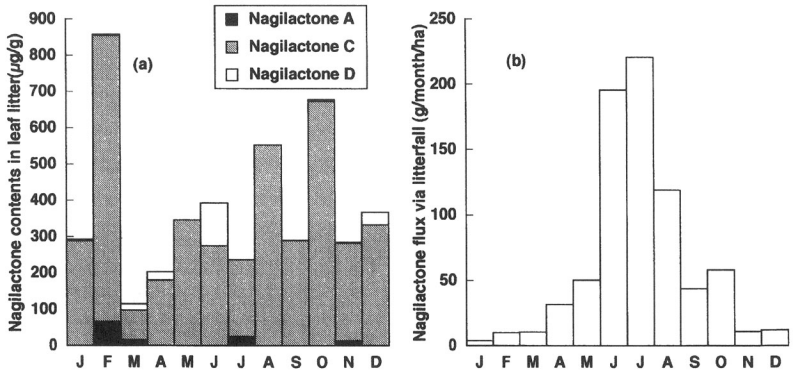

FIG. 2. Seasonal changes in nagilactone content in leaf litter (a) and nagilactone flux via leaf litter (b). The annual average nagilactone content in leaf litter was 3.8×10^{-4} g/g. Nagilactone flux in each month was calculated by multiplication of the mass of podocarp leaf litter with the nagilactone content in leaf litter each month.

nagilactone content (Figure 2b). The seasonal changes in nagilactone transport were parallel to that of litterfall, since the variation in leaf nagilactone concentrations was smaller than that in monthly leaf litterfall. A total of 765.3 g/ha nagilactone is transported within a year. Hence the nagilactone flux by leaf litterfall is very much larger than that of throughfall. To eliminate data fluctuation for the mixture of unusual green leaves with the leaf litter sample, the annual mean of nagilactone contents in leaf litter was not computed by using the average of nagilactone contents in leaf litter, but by dividing this flux by the annual leaf litterfall of 2365.4 mg/ha/yr. This gives an annual mean nagilactone concentration of 323.6 μg/g for leaf litterfall, and this value is equivalent to 84% of the nagilactone concentration in mature green leaves (Ohmae et al., 1996). The difference in concentration between the mature green leaves and leaf litter (16% nagilactone in mature green leaves) is assumed to be the result of the translocation from leaf litter to the mature green leaves before defoliation or the breakdown of nagilactone in leaf litter. In fact, this percentage of translocation is similar to that of nutritional mineral elements so far studied in various forest ecosystems (Tsutsumi, 1987). Based on the translocation rate, 144 g/ha nagilactone is reabsorbed annually from leaves to the tree body before defoliation.

Decrease in Nagilactones from Decaying Leaf Litter. Leaf litter accumulated on the forest floor is a major component of the Ao layer and is a source of inorganic and organic matter including various chemicals such as nagilactone. The accumulation and release of nagilactone in the Ao layer was estimated by the litter bag experiment.

After 12 months, residual litter was 71.5% of the original mass in a litter bag (Figure 3). The decomposition rate of podocarp leaf litter was calculated by using the following exponential curve (Olson, 1963),

$$L/L_0 = exp(-0.33t); \qquad t_{0.5} = 2.1$$

where t is time in years after the initiation of the litter bag experiment, L is remaining litter mass in grams in the litter bag at the given time t, L_0 is initial litter mass in grams in the litter bag, and $t_{0.5}$ is half-life in years for the decaying leaf litter.

After 12 months, residual nagilactone in the bags was only 0.3% of the original leaf litter contents (Figure 3). The rate of disappearance of nagilactone also was calculated by using an exponential curve:

$$C/C_0 = exp(-5.8t); \qquad t_{0.5} = 0.12$$

where C is nagilactone concentrations of leaf litter in litter bags at the given time t, C_0 is the initial nagilactone concentration, and the other symbols are the

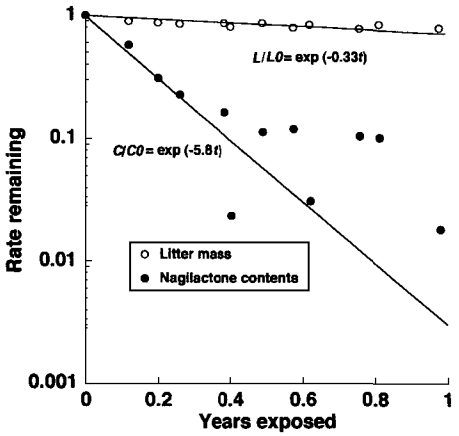

FIG. 3. The decomposition rate of the podocarp leaf litter and the disappearance rate of nagilactones from leaf litter. The time is t in years after the initiation of the litter bag experiment, L is the remaining liter mass in grams in a litter bag at time t, L_0 is the initial leaf liter mass in grams in a litter bag, $t_{0.5}$ is the half life in years for the decaying leaf litter, C is the nagilactone concentration of leaf litter in a bag at time t, C_0 is the initial nagilactone concentration, and $t_{0.5}$ is half life in years.

same as those given above. The rate of disappearance of nagilactones was much greater than the decomposition rate of the leaf litter mass dry weight.

Nagilactones in the Ao Layer. The average Ao layer of dead leaves and small branches was 8.6 ton/ha of dry matter, of which 6.9 ton/ha was dead podocarp leaves. The dead podocarp branches are scarce because podocarp wood decays easily and is not durable. Large podocarp branches seldom fell in our study area and are therefore only expected after catastrophic events such as violent typhoons. The most recent one was in 1961 (Shimoda et al., 1994). The mean concentration of three nagilactones in dead podocarp leaves in the Ao layer wsa 25.7 μg/g. By multiplying this concentration by the 6.9 ton/ha mass of podocarp leaves on the forest floor, we obtain an estimate of 177.3 g/ha nagilactone that is accumulated on the forest floor. This nagilactone quantity is about 5% of that in living mature leaves.

Nagilactones in Surface Soil. If nagilactone inhibits plant growth in situ, its concentration in surface soil is important because surface soil is close to a root mat rich in fine roots available for absorption of water and chemicals. The seasonal changes in nagilactone contents in surface soil at depths of 0–10 cm is shown in Figure 4. The total concentrations of the three nagilactones in soil varies between 0.05 and 0.92 μg/g. The higher concentration in July 1992 implies the rapid release of nagilactone from leaf litterfall, which peaks in June

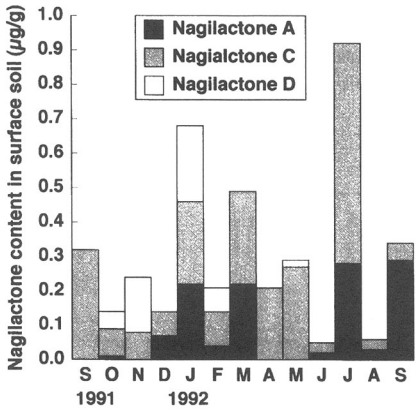

FIG. 4. Seasonal changes in nagilactone content in the soil. The average annual nagilactone content in soil was 3.1×10^{-7} g/g.

and July (Figure 2b). These nagilactone concentrations have no significant correlation to nagilactone contents in leaf litterfall and in throughfall. The proportion of each isomer to the total nagilactone contents was different from that of leaf litter. Nagilactone A was detected in all monthly soil samples during the sampling period, while it was found in leaf litter samples in six of 13 sampling months (Figure 4).

The annual mean content of total surface-soil nagilactones, 0.31 μg/g, was smaller than that in leaf litter in the Ao layer (25.7 μg/g). The mean bulk density of soil from 0–10 cm depth was 0.87 g/ml, while the mean field capacity as a measure of soil water was 0.49 g/ml. These quantities give an estimate of possible nagilactone concentration at an ideal soil water condition of the field capacity. The estimated nagilactone concentration per unit soil water was 0.55 μg/ml or 1.5×10^{-6} M, which is 2.0% of the concentration for 50% growth inhibition of *Avena* coleoptiles (Hayashi and Sakan, 1974). Therefore, nagilactone concentration in surface soil appears to have little effect on plant growth in situ.

Nagilactones at Different Soil Depth. Nagilactone concentrations in mineral soil rapidly decreased with an increase in soil depth and could not be detected in soil over 30 cm depth (Figure 5a). A decrease in soil nagilactone with an increase in soil depth can be approximated by the exponential function of soil depth (Figure 5b) (Ogawa, 1961), as follows:

$$C(z) = 1.4exp(-1.5z)$$

where z is soil depth in centimeters and $C(z)$ is soil nagilactone concentration in

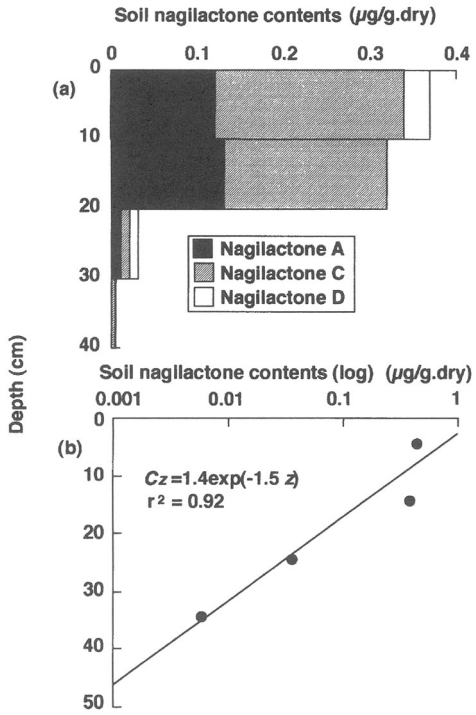

FIG. 5. The vertical distribution of nagilactone content in soil. Nagilactones were transported to a depth of 40 cm (a). The decrease in nagilactone content was approximated by an exponential function (b) in which z is soil depth, $C(z)$ is soil nagilactone content at depth z.

micrograms per gram at soil depth z. The estimated nagilactone concentration in surface soil was 1.4 μg/g, which is 0.43% of the annual mean concentration of leaf litter (323.6 μg/g). By multiplying the mean soil bulk density of 112 g/100 ml, and by integrating the $C(z)$ vs. z relation over the whole range of z [0, infinity], we estimate the nagilactone accumulation in mineral soil to be 105.0 g/ha, which is far smaller than the nagilactone mass accumulated in the living leaf canopy.

The possible water volume in the soil was calculated by using the data of field capacity for the rhizosphere soil at 0–100 cm depth. It was 4.2×10^6 l/ha/100 cm. If 105.0 g/ha nagilactones in mineral soil are all dissolved in the soil water at the rhizosphere, its concentration in the soil water is 0.025 μg/ml or 6.6×10^{-8} M. Therefore, nagilactones in mineral soil are too low to work as a plant growth inhibitor under wet forest soil conditions.

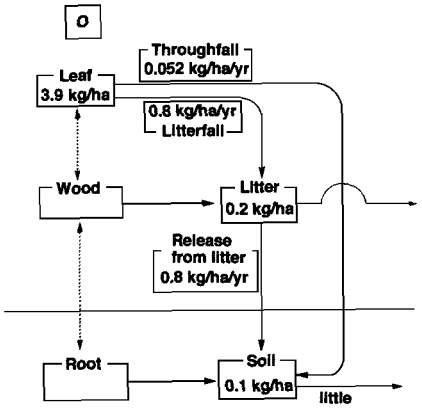

FIG. 6. A diagram of the forest showing the different pools and fluxes of nagilactone.

DISCUSSION

Implications of Nagilactone Flux by Throughfall. The nagilactones are leached out from canopy leaves, dissolved in throughfall, transported to the forest floor, and permeated into the soil (Figure 6). Thus, nagilactones move from a canopy compartment to a forest floor compartment along the pathways of the water cycle in forest ecosystems. Throughfall is highly correlative with incident precipitation.

The nagilactone flux by throughfall was very small (0.052 g/ha/yr) (Figure 6), which resulted from the very low nagilactone concentrations in throughfall. The mean annual nagilactone content in throughfall, 4.7×10^{-12} g/ml, is equivalent to 1.2×10^{-11} M in terms of the largest molecular weight of nagilactone C. This nagilactone content is very much lower than the nagilactone C concentration of 7×10^{-5} M for 50% growth inhibition of *Avena* coleoptiles (Hayashi and Sakan, 1974). Furthermore, this nagilactone content in throughfall is very much lower than that in living mature leaves, which was 3.8×10^{-4} g/g or 1.0×10^{-3} M (Ohmae et al., 1996). Therefore, nagilactone in throughfall does not induce any allelopathic influence on plants in situ.

Ishikawa (1971) investigated the effects of the aqueous extract of podocarp leaves on the germination and the growth of seedlings of some herbaceous species. In that study, two types of aqueous extracts of podocarp leaves were prepared by using two different procedures in sample leaf handling. One extract was obtained by cutting sample leaves into small pieces of 1.0 cm width, while the other was obtained without cutting sample leaves. The aqueous extract from cut leaves strongly inhibited germination and seedling growth. However, the aqueous extract of whole leaves without injuries had little effect. These results

suggested the leaching of nagilactones from living whole leaves into throughfall was weak and explains the low nagilactone concentration in throughfall in the present study.

However, the possibility of nagilactones leaching into throughfall can not be completely ruled out. In fact, the nagilactone content in throughfall had a slight positive correlation with sampling durations and the number of fine days during the sampling period, and it had a slight negative correlation with various rainfall indices. Throughfall is a sum of rain droplets, which have remained on the surface of leaves for a short time. The amount of leached nagilactones depends on the velocity and absolute mass of rain droplet flux on the leaf surface. In conjunction with extraction time, the quantity of nagilactones in throughfall in this study appears to be smaller than that in the aqueous extract prepared by Ishikawa. Therefore, the results of low nagilactone concentration in throughfall in the present study quantitatively support Ishikawa's conclusion, which was that there was no effective physiological activity of aqueous extracts from whole uninjured leaves.

Importance of Nagilactone Flux by Litterfall. Nagilactones also are transferred from canopy leaves to the forest floor by litterfall (Figure 6). The nagilactones transported from the canopy to forest floor via leaf litter varied seasonally and paralleled the changes in podocarp leaf litterfall. Litterfall is the most important transport agent, through which nagilactones are released to the forest floor. The immobility of nagilactones through water cycling appear to be fundamental to understanding nagilactone dynamics in situ.

Rapid Disappearance of Nagilactones from Leaf Litter. Dry matter decomposition rate of podocarp leaf litter per unit initial litter weight per unit time on the forest floor was 0.33/yr in the litter bag experiment. This decomposition rate of leaf litter in dry matter is similar to that of the *Cryptomeria* forest in Yoshino, Nara, about 33 km south of the research plot and within a range of the decomposition rates of soil organic matter in Japan (Tsutsumi, 1987). Precipitation and temperature can affect decomposition rates more strongly than litter quality in regional scales (Vitousek et al., 1994). Therefore, species difference between *P. nagi* and *C. japonica* is negligible.

Nagilactones are either released from or will be decomposed in leaf litter and finally disappear from the litter. The litter bag experiment revealed the very rapid disappearance of nagilactone from decomposing leaf litter. Here, the disappearance rate of nagilactone represents a relative rate, which is defined by the quantity of nagilactone in grams per unit of initial nagilactone that disappeared in grams in leaves per unit time in years. The disappearance coefficient, thus defined, became 5.8/yr, which is 17 times greater than the decomposition coefficient of leaf litter in dry matter (0.33/yr). Therefore, most of the 765.3 g of nagilactones, which are transported from canopy leaves to the forest floor by leaf litter, disappear within a year (Figure 6).

The decomposition process of litter (L) in the Ao layer with respect to time t can be described by a linear differential equation of the first order, $dL/dt = L_0 - kL$, where L_0 is the input of litter per unit time to the Ao layer by litterfall and k is a decomposition coefficient of litter per unit time (Olson, 1963). At the steady state, where $dL/dt = 0$, the variable L is equivalent to L_0/k or $L = L_0/k$. Inserting $L_0 = 2.4$ ton/ha/yr and $k = 0.33$/yr in the present study into the mathematical model, we obtain an expected quantity of 7.2 ton/ha podocarp leaves in the Ao layer that is simlar to the observed quantity of dead podocarp leaves in the Ao layer (6.9 ton/ha). If a decrease in nagilactone in dead leaves with an increase in time follows the same differential equation for dry matter decomposition, the expected nagilactone mass in the Ao layer is obtained by calculating the ratio of nagilactone flux via leaf litterfall (765.3 g/ha/yr) to the nagilactone decrease coefficient (5.8 /yr) in the litter bag experiment. The nagilactones in the Ao layer, thus calculated, were 131.9 g/ha, which is not largely different from the observed nagilactone mass in the Ao layer, 177.3 g/ha (Figure 6). Therefore, the rapid disappearance of nagilactones from litter bags explains the low nagilactone stock in the Ao layer.

Low Stock of Nagilactones in Soil. A decreased pattern of soil nagilactone contents with an increase in mineral soil depth was approximated by an exponential function of soil depth. The quantity of nagilactones accumulated in mineral soil was 105.0 g/ha. The soil water stock at 0–100 cm depth was 4.2×10^9 ml/ha, which was obtained from wet soil during the field capacity state and is equivalent to 420 mm in water height adopted as a rainfall unit. In natural forests, the soil water stock is lower than the value of field capacity except on rainy days. The soil water is the aqueous solution that contains various dissolved chemicals (Nasu and Sakuma, 1997). Therefore, a decrease in the soil water stock means a decrease in solvents. If nagilactone mass is constant, a decrease in the soil water stock raises the nagilactone concentration. The relationship between the soil water density and soil nagilactone concentration is shown in Figure 7. Nagilactones cannot exhibit the allelopathic effect until the soil water density is lower than 0.027 g/cm^3. In natural forests, such a state of soil water will not be observed, and if such a low soil water stock occurred, plants would die for lack of moisture, regardless of the allelopathic effects of nagilactone. Thus, nagilactone in the soil cannot act as an allelopathic substance.

The nagilactone stock in the soil, as a sum of the Ao layer and mineral soil, was 282.3 g/ha. A quotient of the nagilactone stock in the soil to the nagilactone input by leaf litterfall (765.3 g/ha/yr) gives a turnover time for nagilactone, and litterfall is a major pathway of nagilactone flow from the forest canopy to the forest floor. The calculated turnover time is 0.37/yr. It follows that the disappearance of nagilactones in the soil is much faster than that of carbon and other nutritional mineral elements (Tsutsumi, 1987; Nakane, 1979). Nagilactones can not affect germination and seedling growth of *P. nagi* (Yoshida, 1989). There-

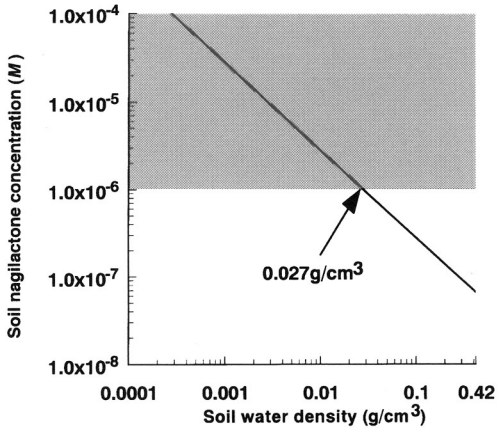

FIG. 7. The relationship between soil water density and soil nagilactone concentration dissolved in the soil water. Nagilactone can exhibit allelopathic effect only when soil water is lower than 0.027 g/cm^3 (shaded area).

fore, nagilactones do not disturb the growth of *P. nagi*, even if nagilactones are selectively absorbed by *P. nagi*. Ohmae et al. (1996) described the possible translocation of nagilactone from mature leaves to new leaves. In the present study, we clarified the immobility of nagilactones via throughfall, reabsorption of nagilactones from leaves before defoliation, rapid disappearance of nagilactone from decaying leaf litter, and low nagilactone stock in soil. None of these findings contradict the proposed active reabsorption of nagilactones by *P. nagi*.

If the active reabsorption of nagilactones by *P. nagi* occurs, the low nagilactone stock in soil should be an inevitable result, which seems to refute the possibility of strong and direct allelopathic effects of *P. nagi* on the growth of other plants in situ. In conclusion, nagilactone cannot work as an allelochemical, which directly inhibits the growth of other plants without borrowing other ecological agencies, but works effectively against the feeding pressure on *P. nagi* from animals (Ohmae et al., 1996) in our study forest.

In addition to *P. nagi*, only three flowering tree species were observed in our research area. Leaf biomass estimates were 10.2 ton/ha for *P. nagi* and 0.5 ton/ha for other flowering trees (Ohmae et al., 1996). Thus, the predominance of *P. nagi* in the study plot is evident. The aforementioned reabsorption hypothesis of nagilactones and rejection of direct allelopathy *P. nagi* to other plants are the conclusions of the present study site where *P. nagi* was dominant. If we consider the study site in the past (Ohmae et al., 1996), when only a small number of podocarp trees were transplanted, the reabsorption mechanisms might not have worked owing to the scarcity of podocarp roots. With regard to this specula-

tion, a high quantity of nagilactone might have accumulated in the soil in the past, resulting in direct allelopathic effects of podocarps on the growth of other plants.

The aforementioned results were obtained from a stabilized forest without significant forest disturbance. If a catastrophic event occurred in such a forest in the future, the results may be completely different. The study forest suffered from forest disturbance caused by repeated typhoons (Naka, 1982; Shimoda et al., 1994). If a typhoon occurred in the forest, many trees would be felled, releasing a large quantity of nagilactone to the forest floor and resulting in a poisonous effect on plant growth. Therefore, nagilactone might have worked as a plant growth inhibitor in situ in the past and may do so again in the future. In other words, the evolutional value of nagilactone might be as an antiherbivory substance in a wet climatic forest environment, but a secondary mechanism could work as a plant growth inhibitor at a time of catastrophic events, such as transplanting or climatic disturbance.

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EFFECTS OF ATTRACTION RADIUS AND FLIGHT PATHS ON CATCH OF SCOLYTID BEETLES DISPERSING OUTWARD THROUGH RINGS OF PHEROMONE TRAPS

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Abstract—Results were analyzed from six previous studies in which marked bark and ambrosia beetles, *Ips typographus*, *I. paraconfusus*, and *Trypodendron lineatum* (Coleoptera: Scolytidae), were released at the center of concentric rings of pheromone traps. Assuming nearly straight flight paths, a "filtering" equation model predicts recapture percentages on several trap rings of specified radii, trap numbers, and effective attraction radius (EAR) of a pheromone trap. Equations were used to calculate recapture percentages on concentric trap rings as a function of increasing EAR and gave polynomial relationships for each ring with terms equal to the number of inner rings plus one. Results were confirmed by computer simulations. Filtering equations were iterated with increasing EAR values to find one that gave a recapture percentage for the innermost trap ring that matched the field results. The estimated EAR for a synthetic pheromone bait of *I. typographus* was similar in five tests (range 1.39–1.78 m), but in two other tests was larger (3.27 and 15.9 m). The EAR for pheromone of 75 male *I. paraconfusus* in ponderosa pine logs ranged from 0.35 to 34.5 m (mean of 4.7 m) and was generally larger for previously pheromone-responding beetles than for freshly emerged ones. For *T. lineatum*, the EAR of lineatin-baited traps at 100-m radius was 2.43 m. Recaptures of *I. typographus* were reasonably predicted by the estimated EARs in the filtering model. To obtain perfect fits, another model assumed the EAR could vary with ring radius (dispersal distance) and found that the EAR for *I. typographus* decreased with dispersal distance in four experiments, but increased or was variable in two others. However, in *I. paraconfusus* and *T. lineatum*, the EAR increased with dispersal distance. Simulations that varied combinations of the EAR and random angles of maximum turning (AMT) of beetles stepwise showed that a nearly straight flight path for *I. typographus* explained observed catches on trap rings best, while a higher AMT of 36° was better to explain catches of *T. lineatum*. Simulations show that catch per trap ring in relation to radial distance can be influenced by the beetle's AMT (still

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unobserved in the field). A conceptual model of dispersal and host selection in "aggressive" bark beetles with regard to pioneer and joiner colonization strategies is presented.

Key Words—Effective attraction radius, dispersion, dispersal, host finding, host selection, Scolytidae, Coleoptera, *Ips typographus*, *I. paraconfusus*, *Trypodendron lineatum*, computer simulation model.

INTRODUCTION

In California, Gara (1963) released marked bark beetles, *Ips paraconfusus*, at the center of one ring of five traps containing ponderosa pine logs infested with 75 males each. The ring radius varied in individual tests from 3 to 2000 m. Since then, several studies in Europe have released marked spruce bark beetles, *Ips typographus*, at the center of several concentric rings of traps releasing pheromone components (Botterweg, 1982; Zumr, 1992; Zolubas and Byers, 1995; Duelli et al., 1997). The striped ambrosia beetle, *Trypodendron lineatum*, also was released from the center of three trap rings baited with the synthetic pheromone lineatin (Salom and McLean, 1989). Duelli et al. (1997) marked 6898 *I. typographus* termed "unflown" (freshly emerged) and 5123 considered "flown" (collected in pheromone traps) and released them at the center of three rings of pheromone-baited traps in a nonhost Scots pine forest (Table 1). Zolubas and Byers (1995) and Zumr (1992) released marked *I. typographus* at the center of four lines of pheromone traps in cardinal directions, which can be considered as concentric rings (only the first four or five rings considered here) in a spruce forest (Table 1). Botterweg (1982) also released marked spruce bark beetles at the center of pheromone trap rings in an area of meadow and Scots pine forest (Table 1). Among the purposes of these studies were to describe how far bark beetles disperse and whether flight behavior or responses to pheromone traps varies with distance from the release point. The authors concluded, based on trap catches, that bark beetles fly away from a release site in all directions when wind speeds are below 1 m/sec.

The "effective attraction radius" (EAR) was proposed as an index of attractive strength for a trap releasing semiochemicals (Byers et al., 1989). Given a population density that is proportional to the unbaited (passive) trap catch, the EAR is the radius that a spherical passive trap would need to be in order to catch, merely by interception, as many dispersing insects as were actually caught on the baited trap. Comparison of catches on passive and pheromone-baited traps gave an EAR of 1.9 m for *I. typographus* response to a release of pheromone components, 50 mg 2-methyl-3-buten-2-ol (MB) and 1 mg (*S*)-*cis*-verbenol (cV) per day (Byers et al., 1989). Similar amounts of MB+cV were also released in

TABLE 1. TRAP RING RADII AND NUMBER OF SYNTHETIC PHEROMONE-BAITED TRAPS PER CONCENTRIC RING IN PREVIOUS STUDIES IN WHICH *Ips typographus* WERE RELEASED AT CENTER OF THE RINGS AND A PORTION RECAPTURED ON PHEROMONE TRAPS

	Ring 1	Ring 2	Ring 3	Ring 4	Ring 5
Duelli et al. (1997)					
Ring radius (m)	5	200	500		
Number traps	4	80	80		
Catch unflown (6898)[a]	2445	650	195		
Catch flown (5123)	2070	95	38		
Zolubas and Byers (1995)					
Ring radius (m)	10 or 30[b]	60	90	120	
Number traps	1 or 4[b]	4	4	4	
Catch (5030 or 5920)[b]	284 or 384[b]	208	64	7	
Zumr (1992)					
Ring radius (m)	50	100	200	300	400
Number traps	4	4	4	4	4
Catch (6600)[c]	2673	1254	719	317	290
Botterweg (1982)					
Ring radius (m)	50	100	200	350	500
Number traps	4	8	16	28	40
Catch (1500 and 7000)[d]	125 or 198[d]	56 or 36	55 or 66	22 or 90	9 or 167

[a]Values in parentheses in first column are number of released *I. typographus*.
[b]Values for experiments 1 and 2, respectively; single values for experiment 2.
[c]1989 and 1990 results pooled.
[d]Experiments 1 (1980) and 3 (1981), respectively.

trap ring studies using Pheroprax baits (Zumr, 1992; Duelli et al., 1997) and Ipslure baits (Botterweg, 1982; Zolubas and Byers, 1995). For simulation studies, the EAR can be considered more simply in two dimensions rather than three because at large EARs the ground and beetle's flight height essentially flatten the theoretical sphere into a cylinder.

In the dispersal studies above, catches on pheromone traps decreased as a function of distance from the release point as described by power and exponential regressions (Zolubas and Byers, 1995). This is expected based on the movement of beetles outward into increasingly greater areas. However, the relationships can be greatly affected by several factors; for example, the EAR of a trap could change with flight distance (proportional to distance from release site). Another factor, previously ignored, is that there is a "filtering" effect such that some beetles would be caught on the first rings of traps while those remaining would pass through possibly to be caught on outer rings. There could also be a selective catch of pheromone-responding beetles on the inner rings while unresponsive beetles would pass through to be caught on traps of the outer rings by chance interception. Bark beetles might even change their angle of turning (or frequency of turning) with flight distance from the source, which ought to

affect catch rates. The previous studies were done in spruce, pine, or Douglas-fir forests so interceptions by trees, expected every 67 m for a 70-year-old Norway spruce forest of 30-cm-diameter trees (Byers, 1996a), might affect the dispersal directions when beetles that had landed took flight again in random directions.

My first objective was to develop equations that can calculate the theoretical filtering effect of any arrangement of concentric trap rings of specified dimensions, numbers of traps, and attractive power (EAR) in order to predict the catch of insects dispersing outward from a central release site. A second objective was to determine the influence of trap EAR and the beetle's angle of maximum turning at random (AMT) on catches of scolytid beetles in various trap ring arrangements in computer simulations. The comparison of predicted catches by using equations and simulations with those catches observed in the field in the previous studies may provide insights concerning the behavior of bark and ambrosia beetles during the initial dispersal from brood trees and overwintering sites. Finally, I develop a theory that during dispersal and host seeking, a beetle exhibits either a "pioneer" or a "joiner" strategy of colonization behavior that is based on competition, host resistance, presence of aggregation pheromone, and the bark beetle's fat reserves.

METHODS AND MATERIALS

Sequential Equations to Predict Catches. A general series of equations can predict the catch on any number of concentric rings of traps depending on the respective ring radii, number of traps per ring, and radii of the traps (assumed to be equal). Considering three rings as in Duelli et al. (1997), the number caught on rings 1–3, C_1–C_3, and the number escaping each ring, E_1–E_3, can be calculated by three successive pairs of equations, assuming a nearly straight flight path (no beetles can come back once they have left a ring):

$$C_1 = N \frac{T_1(EAR)}{R_1\pi} \qquad E_1 = N \frac{R_1\pi - T_1(EAR)}{R_1\pi} \tag{1}$$

$$C_2 = E_1 \frac{T_2(EAR)}{R_2\pi} \qquad E_2 = E_1 \frac{R_2\pi - T_2(EAR)}{R_2\pi} \tag{2}$$

$$C_3 = E_2 \frac{T_3(EAR)}{R_3\pi} \qquad E_3 = E_2 \frac{R_3\pi - T_3(EAR)}{R_3\pi} \tag{3}$$

where N is the initial number of insects released, T_1–T_3 are the number of traps in rings 1–3, EAR is the effective attraction radius of the pheromone trap, and R_1–R_3 are the radii of trap rings 1–3, respectively. The catch on a fourth

ring, or more, can be considered by adding a fourth equation, or more, as indicated.

The sequential equations were used repeatedly by computer to graph the effect of changing the EAR on the percentage of released beetles caught on each trap row. The best-fitting EARs to the data of four studies and various experiments were found by incrementing the EAR from 0 to the maximum possible without overlap (MAX) in steps of MAX/10,000 m using sequential equation 1 by computer to find the least difference in the actual percent catch on ring 1 compared with the predicted catch. This EAR was then used to calculate the predicted catches on the outer rings for comparison to observed catches. Assuming, however, that the EAR can vary with distance from the center, an optimal EAR was found for each ring based on the maximum number that could have passed through the inner rings (by subtracting the catches from the number released) and again on the dimensions and trap numbers of the ring (as calculated for the first ring above).

Simulations to Predict Catches. Insect flight movement can be simulated in two dimensions by taking steps in a forward direction with possible random deviations up to an angle of maximum turn (AMT), either right or left at random (Skellam, 1973; Byers, 1991, 1993a, 1996a,b). An insect is caught when intercepting a trap, no matter how large the step size, according to the algorithm in Byers (1991). A computer model was made to simulate the trapping designs used in previous studies, e.g., by Duelli et al. (1997). The input parameters of the program are dispersal time, number of released insects, average insect speed, step size, AMT, number of trap rings, number of traps per ring, radii of trap rings, and the EAR of a trap. The release site is centered on the screen, and coordinates of the traps are calculated and traps drawn. Insects are given random initial directions (random number 0–360°).

In all simulations, flight speed was 2 m/sec, which is about what large bark beetles such as *Ips typographus* can maintain in still air (Byers, 1996a). Simulated dispersal periods were limited to 1 hr when AMT was varied, although a few beetles on flight mills have flow up to 6 hr (Forsse and Solbreck, 1985; Forsse, 1991; Gries et al., 1990). The AMT was varied from 0° to 45°, and steps were 2 m (possible turn every second). Catch was recorded for each trap ring. The "flown" bark beetles had a catch distribution on the three rings that was different from the "unflown" (Duelli et al., 1997) and did not fit well to the predicted based on the best-fitting EAR calculated for ring 1. Therefore, the EAR and the AMT were varied two-dimensionally (AMT varied at each varied EAR) in an attempt to find an EAR–AMT combination of simulated results that could predict the field data. The EAR was the same for all traps in the rings. Other catch distributions (Zolubas and Byers, 1995, *I. typographus;* Salom and McLean, 1989, experiment 1, *T. lineatum*) were modeled similarly.

The expected catch per trap with an EAR of 1.9 m at increasing distances

from the release point of 1000 insects was estimated by simulation when only one trap was present (no filtering effect). Insects had either an AMT of 5° or 20° and flew for 30 min. In another series of related simulations, competing traps were placed in 10 concentric rings of 20, 10, and then 4 traps each, respectively, every 25 m, with the same EAR and an AMT of 5°. Nonlinear regressions were fitted to the data when appropriate.

RESULTS

Sequential Equations to Predict Catches. The computer-iterated equations 1–3 found that increasing the effective attraction radius (EAR) of a trap from 0 to the maximum, without overlap between traps, linearly increases the recapture percentage (of those released) on trap ring 1 [for the trap configuration of Duelli et al. (1997), $Y = 25.465X$, $r^2 = 1$, Figure 1]. The innermost trap ring always will have a linear increase in recapture percentage as a function of EAR, regardless of the ring radius or number of traps in the ring. The recapture percentage on the second ring of traps has a quadratic relationship to EAR, first increasing then decreasing (Figure 1), again regardless of the ring radii or number of traps. For the trap configuration of Duelli et al. (1997), the relation follows $Y = -3.242X^2$

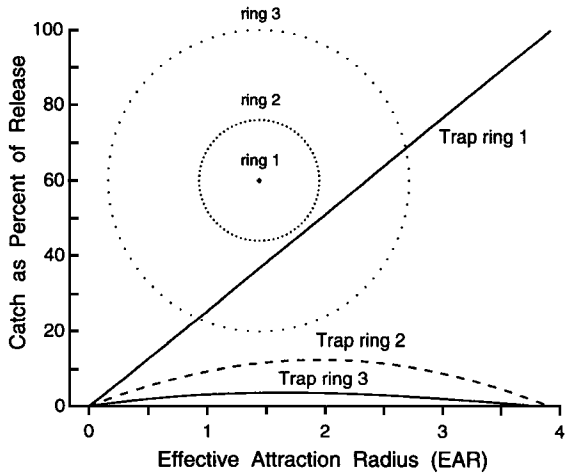

FIG. 1. Percentage caught on each trap ring of the number of insects released in relation to the effective attraction radius (EAR) of the trap. The curves are based on equations 1–3 for four traps in ring 1 (5 m radius), 80 traps in ring 2 (200 m radius), and 80 traps in ring 3 (500 m radius). The maximum trap radius of 3.927 m is based on four nonoverlapping traps in ring 1. Concentric rings of dots represent trap ring dimensions and number of traps (3 m radius).

TABLE 2. PERCENTAGES OF RECAPTURED *Ips typographus* IN PREVIOUS STUDIES COMPARED WITH PREDICTED PERCENTAGES CALCULATED FROM FILTERING MODEL WITH AN EFFECTIVE ATTRACTION RADIUS (EAR) OF PHEROMONE TRAPS BEST FITTING THE OBSERVED PERCENTAGE FOR TRAP RING 1

	Percentage recaptured				
	Ring 1	Ring 2	Ring 3	Ring 4	Ring 5
Duelli et al. (1997)					
Catch unflown (6898)	35.45	9.42	2.83		
Predicted (1.39 m EAR)	35.45	11.44	3.77		
Catch flown (5123)	40.41	1.85	0.74		
Predicted (1.59 m EAR)	40.41	12.04	3.84		
Zolubas and Byers (1995)					
Catch (5030 or 5920)[a]	5.65 or 6.49[a]	3.51	1.08	0.12	
Predicted (1.78 or 1.53 m EAR)[a]	5.65 or 6.49[a]	3.03	1.96	1.44	
Zumr (1992)					
Catch (6600)	40.5	19.0	10.89	4.8	4.39
Predicted (15.9 m)	40.5	12.05	4.8	2.88	2.01
Botterweg (1982)					
Catch (1500 or 7000)[b]	8.33 or 2.83[b]	3.73 or 0.51	3.67 or 0.94	1.47 or 1.29	0.6 or 2.39
Predicted (3.27 or 1.11 m EAR)[b]	8.33 or 2.83[b]	7.64 or 2.75	7.0 or 2.67	6.42 or 2.6	5.88 or 2.52

[a]Values for experiments 1 and 2, respectively; single values for experiment 2.
[b]Experiments 1 (1980) and 3 (1981), respectively.

+ $12.732X$, $r^2 = 1$ (Figure 1). The third ring of traps catches insects similarly (Figure 1) but even less according to a cubic relationship, $Y = 0.165X^3 - 1.945X^2 + 5.09X$, $r^2 = 1$. Trap placements with four or more rings are related as polynomials of four or more terms; however, the specific coefficients depend on the actual EAR, ring radii, and number of traps per ring.

The EAR of pheromone-baited traps in previous studies can be estimated using sequential equation 1 and incrementing the EAR until the predicted catch percentage matches the observed catch. Trap ring 1 is the only reliable one to use since the relation is monotonic, while rings 2 and 3 are unimodal with two values of X for each Y (Figure 1). In the study by Duelli et al. (1997), the pheromone traps would need to have an EAR for "unflown" *I. typographus* of 1.39 m to account for the observed catch of 35.45% recaptures on ring 1 (Table 2). This value also predicts recapture percentages on rings 2 and 3 that are quite close

to observed values. The best-fitting EAR for the "flown" beetles was 1.59 m yielding 40.41% catch on ring 1 as observed, but the predicted catches on rings 2 and 3 of 12.04 and 3.84% are much larger than the observed values of 1.85 and 0.74%, respectively (Table 2). The sequential equations predict a similar EAR of 1.78 or 1.53 m in the studies by Zolubas and Byers (1995) and 3.27 or 1.11 m in two studies by Botterweg (1982), and even the recapture rates on rings 2–5 are similar to predicted catches with some exceptions (Table 2). The EAR calculated for traps used by Zumr (1992), however, is much larger at 15.9 m, but the predicted catch rates are reasonably similar to those observed, again with some unexplained deviations (Table 2).

In contrast to a constant EAR assumed above, it is possible that bark beetles change their responsiveness to pheromone as they fly away from the release site (or as a function of flight time), thereby resulting in a variable EAR. The same result could occur if bark beetles varied inherently in their responsiveness to pheromone so that more pheromone-sensitive individuals would be filtered out by the inner rings of traps. The best-fitting EAR can be predicted based on the number expected to pass through each succeeding ring. The EAR for *I. typographus* appears to decline significantly with distance of dispersal from the release (Table 3), for example, from 1.59 to 0.15 m (Duelli et al., 1997) and from 1.53 to 0.11 m (Zolubas and Byers, 1995) or from 3.27 to 0.24 m in experiment 1 of Botterweg (1982). However, the EAR was consistently large (11–17 m) in Zumr (1992), but went down and then increased again with trap ring distance in experiment 2 of Botterweg (1982).

TABLE 3. ESTIMATED EFFECTIVE ATTRACTION RADII OF SYNTHETIC PHEROMONE TRAPS FOR EACH CONCENTRIC TRAP RING OF PREVIOUS STUDIES IN ORDER TO OBTAIN OBSERVED CATCHES OF *Ips typographus* WITH FILTERING MODEL

	Effective attraction radius (EAR) (m)				
	Ring 1	Ring 2	Ring 3	Ring 4	Ring 5
Duelli et al. (1997)					
Unflown	1.39	1.15	1.01		
Flown	1.59	0.24	0.25		
Zolubas and Byers (1995)					
Experiment 1 or 2[a]	1.78 or 1.53[a]	1.77	0.85	0.12	
Zumr (1992)					
Pooled release	15.9	25.1	42.3	38.2	55.7
Botterweg (1982)					
Experiment 1 or 3[b]	3.27 or 1.11[b]	1.6 or 0.21	1.64 or 0.38	0.68 or 0.53	0.28 or 0.99

[a]Values for experiments 1 and 2, respectively; single values for experiment 2.
[b]Experiments 1 (1980) and 3 (1981), respectively.

TABLE 4. ESTIMATED EFFECTIVE ATTRACTION RADII (EAR) OF TRAPS WITH NATURAL PHEROMONE OF *Ips paraconfusus* (75 MALES IN LOGS) FOR NINE EXPERIMENTS OF VARIOUS TRAP RING RADIUS TO OBTAIN OBSERVED CATCHES (GARA, 1963)[a]

Ring radius (m)[a]	Emerging beetles		Responding beetles	
	Recapture %	EAR [m (95% CL)][b]	Recapture %	EAR [m (95% CL)]
3	18.58	0.35 (0.31–0.40)	19.05	0.36 (0.32–0.41)
5	28.18	0.89 (0.80–0.97)	26.73	0.84 (0.77–0.92)
10	20.62	1.30 (1.14–1.47)	25.92	1.63 (1.35–1.95)
25	15.74	2.47 (2.22–2.74)	16.96	2.66 (2.44–2.91)
50	5.97	1.88 (1.31–2.67)	16.39	5.15 (4.45–5.93)
100	2.31	1.45 (0.57–3.64)	11.89	7.47 (5.22–10.52)
500	1.45	4.56 (2.20–9.27)	10.98	34.49 (29.72–39.93)
1000	0.70	4.40 (1.19–15.71)	1.27	7.98 (4.21–15.08)
2000	0	—	0.13	1.68 (0.25–9.30)

[a]Five traps equally spaced in ring.
[b]Confidence limits for proportions (from Gara, 1963) were used to calculate 95% confidence limits for EAR.

In the study with *I. paraconfusus* (Gara, 1963), nine experiments had a trap ring of radii from 3 to 2000 m (five traps in a ring) with EARs estimated to range from 0.35 to 34.5 m, average of 4.68 ± 4.13 m (95% CL, Table 4). Beetles that had freshly emerged were marked with one color of fluorescent powder, while another group that had responded previously to pheromone was colored differently, and both groups were released simultaneously. The EARs for both groups increased similarly as trap rings were enlarged from 3 to 25 m radius, then the EAR for the emerged group increased little with increases in ring radii while the EAR for the previously responding group continued to increase (Table 4). *T. lineatum* released in three concentric rings of traps (Salom and McLean, 1989) had EARs that increased from only 0.32 m close to the release to 1.72 m at 100 m radius (Table 5). In the one-ring tests, the EAR also seems to increase up to 7.1 m at 500 m radius (Table 5).

Simulations to Predict Catches. The catch of simulated insects dispersing outward through rings of traps (Figure 2) verified the sequential equations (Table 2, unflown) when the flight path was nearly straight, meaning that the angle of maximum turn at random (AMT) was only a few degrees. An extension of the AMT to 90° caused paths to twist wildly, and this caused the relationships between the AMT and the recaptured percentage on the various rings to be complicated (Figure 3). It was thought that simulations varying both the EAR and AMT could find values that would predict the percentages of catch of "flown" *I. typographus* on the various rings that were not fitted well by the equation model

TABLE 5. ESTIMATED EFFECTIVE ATTRACTION RADII OF LINEATIN-BAITED TRAPS FOR EACH CONCENTRIC TRAP RING TO OBTAIN OBSERVED CATCHES OF *Trypodendron lineatum* WITH FILTERING MODEL (SALOM AND MCLEAN, 1989)

	Effective attraction radius (EAR, m)		
	Ring 1	Ring 2	Ring 3
Experiment 1[a]	0.32 (0.30–0.34)[b]	1.04 (0.98–1.10)	1.72 (1.61–1.84)
Experiment 2[c]	2.43 (2.29–2.59)		
Experiment 3[d]	7.07 (5.11–9.74)		

[a]Trap ring radii of 5, 25, and 100 m with 4, 8, and 16 traps for rings 1 to 3, respectively; and recaptures of 8.1, 9.7, and 7.2% per trap ring, respectively, of 10,535 released in six replicates.
[b]Confidence limits for proportions were used to calculate 95% confidence limits for EAR.
[c]One trap ring of 16 traps in a radius of 100 m, and recapture of 12.5% of 6780 released in four replicates.
[d]One trap ring of four traps in a radius of 500 m and recapture of 35 of 1985 released.

(Table 2). However, none of the stepwise values of EAR from 0 to 3.93 m (maximum without overlap) while varying AMT from 0 to 90° was able to predict the distribution of catch percentages of "flown" beetles on the three trap rings of Duelli et al. (1997). For example, using an EAR of 1.39 m and varying the AMT (0–90°) shows that no relative catch distributions on the three rings were

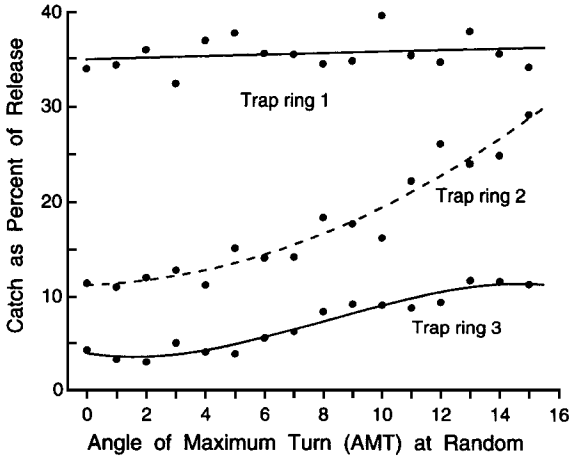

FIG. 2. Percentage caught on each trap ring of the number of insects released in relation to the insect's angle of maximum turn (AMT) at random, assuming a 1.39-m effective attraction radius (EAR) for a trap. The curves are based on release of 1000 simulated insects for each X value with trap arrangement as in Figure 1 (see text for details).

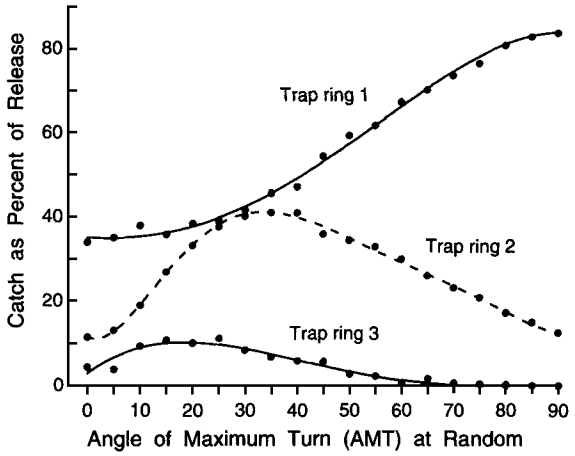

FIG. 3. Percentage caught on each trap ring of the number of insects released in relation to the insect's angle of maximum turn (AMT) at random, assuming a 1.39-m effective attraction radius (EAR) for a trap. The curves are based on release of 1000 simulated insects for each X value with trap arrangement as in Figure 1 (see text for details).

similar to those found in the field (Figure 3, Table 2). The "best" fit, although unsatisfactory, was an AMT of 0° or straight flight path.

The best fit for the data of Zolubas and Byers (1995) for each ring was better, giving an EAR of 1.6 m, an AMT of 0°, and recapture percents of 6.3, 3.5, 0, and 0% per ring, respectively (observed were 6.5, 3.5, 1.1, and 0.1%). By using the data of Salom and McLean (1989) for *T. lineatum*, an EAR–AMT combination was found that fit the observed catch percentages best with an AMT of 36° and EAR of 0.2 m, giving percentages of 8.5, 9.4, and 8.0% (compared to the observed 8.1, 9.7, and 7.2% recapture). A flight duration of only 10 min did not change the results much, as the best EAR was 0.3 m and AMT was 36°. This method does not work for experiments with only one ring. For example, in the second experiment, they recaptured 12.4% on the 16 traps in a ring of 100 m radius (Table 5), which in simulations was fit by many combinations of EAR–AMT, from an EAR of 0.2 m and AMT of 38° to an EAR of 2.5 m and AMT of 0°. In this case, the EAR and AMT vary inversely (in a negative logarithmic relation: EAR = 14.78 − 13.27 ln AMT, r^2 = 0.97), and thus no conclusions can be drawn about flight paths.

The catch per trap as a function of trap distance from the release site has been plotted in most earlier studies, as summarized by Zolubas and Byers (1995). Simulations which used an ideal situation of only one trap, so that competition among traps could not occur, showed a power relationship between catch and

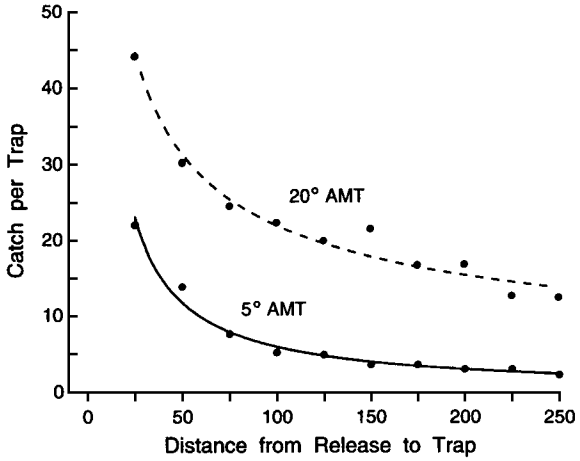

FIG. 4. Catch on one trap as a function of distance from the point of insect release at two different random angles of maximum turn (AMT). Each point represents an average of 10 simulations of 1000 insects each that flew for 30 min. The effective attraction radius of the trap was 1.9 m.

distance ($Y = 540.19X^{-0.98}$, $r^2 = 0.98$) that depended on the AMT (Figure 4). For example, a more twisting AMT of 20° causes a higher catch on the trap at all distances, compared to a more straight path with an AMT of 5° (Figure 4). When traps were competing and filtering the beetles with flight paths of 5° AMT, the catch per trap was less, as expected, compared to the situation with only one trap (Figure 5). However, the first ring of traps filtered out many beetles, which biased the catch on the second ring so that it did not fit the general curve compared to the outer rings (Figure 5). This effect is evident in earlier studies where many beetles were caught on the first ring (Figure 7 of Botterweg, 1982; Figure 2 of Duelli et al., 1997) but is minimal in other studies where only four traps per ring were used at farther distances (Zumr, 1992; Zolubas and Byers, 1995). This effect tends to confound the regressions in a way that was not realized earlier (Zolubas and Byers, 1995).

DISCUSSION

The sequential equations used to filter out the dispersing insects on successive rings of traps were validated by simulation and, in some cases, by a good match with field catches. Usually, the traps in a ring, both in the simulations and in the field, are spaced equally apart. However, it does not matter whether the traps are placed at random or spaced about the ring as long as there is no

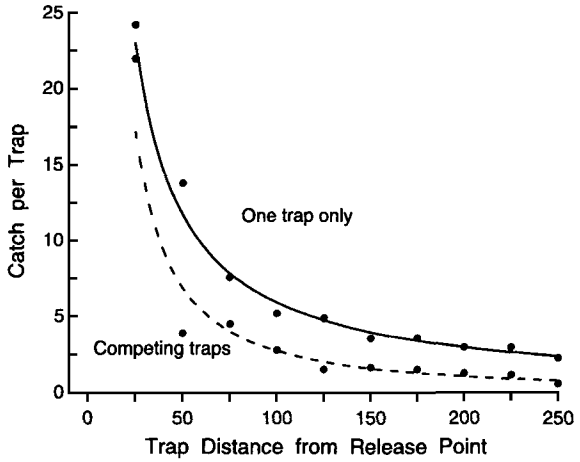

FIG. 5. Comparison of catch per trap in simulations with only one trap versus many competing traps in rings at indicated distances from the release site. The number of competing traps in each successive ring was 20, 10, and then constant at four traps per ring. Each point represents an average of 10 simulations of 1000 insects each, AMT of 5°, and EAR of 1.9 m, with flight for 30 min.

overlap of the EAR (or plume) and the beetles disperse in all directions equally at random. It also does not matter if beetles fly in one general direction (e.g., downwind) with random deviations; nonoverlapping EARs of traps theoretically would filter in the same proportions. In the simulations, on the other hand, if insects fly completely straight (0° AMT), then inner traps prohibit outer traps along the same trajectory from catching (which did not happen in any studies). Therefore, simulated traps must be offset or the insects must have some degree of random turning. However, too much random turning will cause them to turn back occasionally into a ring of traps they have already passed through, thereby possibly inflating the catch on that ring. The sequential equations do not consider the AMT or trap placement and, thus, give ideal results. The equations should not be used with an EAR that overlaps with other traps. In nature, the EAR could overlap but this should decrease catches as the traps directly compete and the insects could be confused by background levels of pheromone. Gara (1963) showed that bark beetles would fly past sources of natural pheromone when overlapped by pheromone from sources upwind.

The EAR is expected to be smaller than an envisioned average distance of oriented flight toward a semiochemical source, which, in turn, is likely smaller than the average distance that bark beetles would first detect such a source (Byers et al., 1989; Schlyter, 1992). One can imagine a pheromone plume as globules

and filaments of higher and lower (or no) pheromone concentration snaking, splitting, or exploding into larger and more uniform clouds that dissipate below the threshold detection of the insect (of. Byers, 1996b). The probability that an insect will orient to the pheromone source after entering this plume depends in large part on the entry point in relation to the source. It is obvious that all these probabilities, behavioral variations, and differences in wind turbulence over time make the calculation of an average orientation distance virtually impossible. What the EAR attempts to do instead is reform the plume and all the orientation probabilities into a sphere (or cylinder) where 100% orient to the source (Byers et al., 1989; Byers, 1995, 1996a).

The EAR for a specific semiochemical release rate and insect species is, in theory, independent of the population density (or number released). Temperature and wind could have some effect by influencing orientation behavior, but this has not been studied. Most behavioral tests are done under similar weather conditions when insects can fly. The EAR can be estimated by comparison of a passive trap catch with the semiochemical trap catch and by using the dimensions of the passive trap (Byers et al., 1989). This method does not depend, in theory, on the trap efficiency, as a lower efficiency is like a lower population density. Neither should change the ratio of catches between the pair of traps. Alternatively, the EAR can be estimated with a second method by using the filtration model, as done here, by comparing the catches on semiochemical-baited traps with the number released from the center. However, in this case, the trap efficiency would affect the EAR.

Increasing the dosage of semiochemical release in traps should give increasing EARs, until inhibition at the highest rates. According to Schlyter et al. (1987), 1 m of Pheroprax tape one week old releases 50 mg MB and 1 mg cV per day (used by Duelli et al., 1997). Zolubas and Byers (1995) used Ipslure baits that released the same rates but also released ipsdienol (probably inactive; Schlyter et al., 1987). Botterweg (1982) used 0.25 m of Pheroprax and Zumr (1992) used some unspecified portion thereof. These studies all appear to have used comparable rates, so the much higher EARs of Zumr (1992) are probably not explained by release rate (Table 3). However, his was the only study that used a cluster of four traps as "the trap." This would both increase the trap surface area by four and broaden the spatial distribution. Byers et al. (1989) showed that enlargement of the sticky trap radius logarithmically increased trap catch of *I. typographus*. The EAR, as estimated with the filtering equations, depends on the trap efficiency, which probably differed in each study: Duelli et al. (1997) used Theysohn traps for ring 1 and a mixture of these and Olesnik traps in outer rings; Zolubas and Byers (1995) used cross-vane barrier traps, while Botterweg (1982) used drainpipe traps. There is no consistent effect of nonhost Scots pine or host Norway spruce forest on the recapture rates in the studies with *I. typographus*.

By using paired sticky traps, an EAR of 1.9 m was calculated for a MB+cV

release (Byers et al., 1989) that is similar to most of those estimated here for the inner trap ring (Tables 2 and 3). An effective catch radius (the same as the EAR) of about 2 m for a puddle trap, releasing the same MB+cV rate, in a grid of 49 (7 × 7) such traps at 6 m spacing was calculated for *I. typographus* (Byers, 1993b). The calculation was based on comparing the ratios of catch on the outer rectangular ring of 24 traps with the next inner ring of 16 traps with simulated results iterating larger EARs. The estimated EAR varied on different dates from 1.53 to 2.48 m (Byers, 1993b). All estimates of EAR which used different traps and methods were similar for the MB+cV release rate. However, in the experiments with multiple trap rings (*I. typographus*), the EAR appeared to decrease with distance (Table 3). This is probably due to a selective catch of responsive beetles on the inner trap rings, leaving less responsive ones caught less often on the outer rings (smaller EAR) rather than to changes in response with flight time.

In contrast, the EAR for *T. lineatum* attraction to lineatin (dose unspecified) in multiple funnel traps had the opposite trend, increasing from 0.32 to 1.72 m (Table 5). The low EAR value at 3 m from the release center may be the result of overlapping of plumes (or EARs) of the closely spaced traps, so that their locations were obscured. When single-ring tests were done at 100 and 500 m radius, the EAR still appeared to increase with radius or dispersal distance (Table 5). The EAR for *I. paraconfusus* to natural pheromone also increased with distance (Table 4). Since there was only one ring in each test, and thus no filtration, the increase in EAR would seem to be a function of dispersal flight distance. The initial increase in EAR close to the release center may result not only from overlapping plumes (or EARs) of the closely spaced traps but also from escape reactions as the beetles were ejected into flight mechanically (as opposed to the other studies where the beetles initiated flight at will). The EAR of 34.5 m seems to be an outlier. Both the "previously responding to pheromone" and "freshly emerged" groups of beetles behave similarly, at least out to 25 m, due to the "overlapping and fright" hypothesis. Then, the EARs enlarge for the previously responding beetles since they appear more willing to respond on average than the freshly emerged beetles that are in the dispersal stage with presumed higher fat reserves.

At the beginning of a dispersal flight, bark beetles are considered rather unresponsive to pheromone or host volatiles. The theory is that fat reserves are higher in freshly emerged beetles so that they have the ability for extended flight and can gain adaptive benefits from dispersal before responding to hosts (Borden et al., 1986; Anderbrant et al., 1985; Gries et al., 1990). Graham (1959) showed that continued flight exercise by *T. lineatum* caused an increase in responsiveness to visual and olfactory stimuli of the host. Freshly emerged *T. lineatum* and *D. pseudotsugae* required 30 or 90 min of flight, respectively, before responding to pheromone from female frass (Bennett and Borden, 1971). Atkins (1966)

found that female *D. pseudotsugae* with more than 20% fat (dry weight) were usually not responsive to the host, while those under 20% fat were responsive and still could fly. Beetles with less than 10% fat had trouble flying since fat was required as an energy source (Atkins, 1969). The fat metabolized by *D. pseudotsugae* consists mainly of C_{16} and C_{18} fatty acids (Thompson and Bennett, 1971). Other studies have found that scolytid beetles in the genera *Trypodendron*, *Dendroctonus*, *Scolytus*, and *Ips* increase their responsiveness or upwind orientation to host and pheromone after continued flight exercise (Choudhury and Kennedy, 1980; and cf. Borden et al., 1986).

However, some bark beetles appear rather responsive to pheromone upon emergence. Lindelöw and Weslien (1986) found that overwintered *I. typographus*, taken from emergence tents in the field and marked, were caught in synthetic pheromone traps within minutes of release. Moreover, the majority of *I. paraconfusus* will respond to aggregation pheromone soon after emergence (Wood and Bushing, 1963; Gara, 1963; Hagen and Atkins, 1975). Botterweg (1982) also found that *I. typographus* can respond immediately to pheromone when beginning dispersal, and this is in accordance with his finding that beetles lost 40–50% of their fat over the winter. Possibly, second-generation beetles in southern Europe would have higher fat and disperse further.

Increasing competition among larvae due to increasing densities of parents laying broods was shown to reduce size and fat content of bark beetles (Atkins, 1975; Anderbrant et al., 1985). This seems in conflict, however, with the statement of Forsse (1991) that flying time of *I. typographus* on flight mills was "similar among populations and appeared unaffected by outbreak conditions." Earlier, Forsse and Solbreck (1985) could not find any affect of sex or body size on the duration of flight on mills. Botterweg (1982) also concluded that there was little, if any, effect of beetle size or fat content on dispersal distance, as monitored in field traps. However, he did find that fat content of beetles declined over the flight period. This was probably due to consumption of fat during host-seeking rather than later emergence of lower-fat beetles, since beetle's sizes (elytral weights) did not decrease over the spring season.

Newly emerged *D. pseudotsugae* fly on flight mills an average of 2 hr before resting (3 hr total) but some individuals can fly up to 8 hr uninterrupted (Atkins, 1961). Jactel and Gaillard (1991) flew *I. sexdentatus* on rotary flight mills and found that 50% of the beetles could fly more than 20 km and 10% more than 45 km based on about 50 interrupted flights. About 25% of *I. typographus* taken from litter in an outbreak area can fly for over 1 hr and 10% for more than 2.5 hr on flight mills, with a maximum flight of 6 hr and 20 min recorded (Forsse and Solbreck, 1985). At free-flying speeds of 1.9–2 m/sec (Gries et al., 1989; Byers, 1996a), a maximum range would be 41 to 45.6 km without wind transport. However, wing beat frequency declines with flight duration, which may affect flight range. In the only case studied, the wing beat frequency of *D. pseudotsugae*

of about 95 Hz declines 18% with flight time over 4 hr to about 75 Hz (Atkins, 1960). Speed on flight mills also declined from 1.11 to 0.99 m/sec (Atkins, 1961).

The view that bark beetles can fly some tens of kilometers is based less on mark–recapture studies and more on collections of beetles far from forests. Nilssen (1978) found two *I. typographus* in the stomach of a salmon 35 km from spruce forest. Miller and Keen (1960) report results of studies by the US Forest Service in California where the western pine beetle, *D. brevicomis*, infested "islands" of ponderosa pine, initially free of beetles, that were separated from the main forest by open sagebrush areas. They concluded that significant numbers of bark beetles must have flown a minimum of 3.2 km in one study, and 9.6 or even 20 km in another study, to reach the infested trees and kill them.

At some point during the flight, or throughout the flight, beetles respond to pheromone, avoid unsuitable trees, or land on trees and presumably determine their suitability. Encountering pheromone while flying, by definition, means that a suitable host is nearby. Other volatiles, notably ethanol (a diseased or decaying tree) and verbenone (signaling competition, fully colonized host, or decaying host), as well as green-leaf alcohols are avoided in flight and after landing (cf. Byers, 1995; Borden et al., 1997; Byers et al., 1998). Some bark beetles respond to host volatiles (e.g., *Tomicus;* Byers, 1995) but the aggressive, tree-killing bark beetles considered here are believed to find hosts by random landing without the aid of any long-range host volatiles (Raffa and Berryman, 1979; Moeck et al., 1981; Byers, 1995, 1996a). For example, *Scolytus ventralis* in one area made borings in 74% of the grand fir, but only 3.5% of the trees were colonized (Berryman and Ashraf, 1970). Hynum and Berryman (1980) also found no differences between landing rates on killed and nonkilled lodgepole pine or between host and nonhost trees for *D. ponderosae*. There was a direct relationship between the magnitude of the flying population (as measured by total catch) and the number of trees landed upon (catch in window traps), indicating a random landing pattern (Raffa and Berryman, 1979). Ponderosa pines that were injured by freezing were as likely to be landed upon by *D. brevicomis* as healthy control trees (Moeck et al., 1981).

A beetle that lands on a tree and attempts to find a place on the bark to bore is termed a pioneer if there are few others present. Pioneers are presumed to encounter significant host resistance and resin when attacking compared to later arrivals (joiners) when the tree has succumbed (Raffa and Berryman, 1979; Wood, 1982; Byers, 1995). Only males, in the case of *Ips*, or females, in the case of *Dendroctonus*, initiate the entrance tunnel and can be pioneers, but the joining sex in the early stages of colonization must incur some increased risks of resinosis. One hypothesis is that, since pioneers must attack the tree and survive to produce pheromone before the rest of the population can exploit the resource, pioneers must be the largest and most vigorous beetles of the population. In

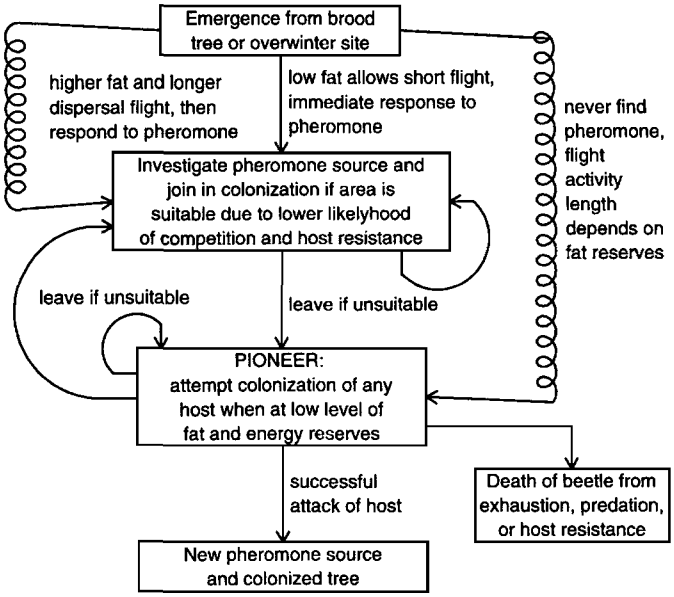

FIG. 6. Conceptual model of dispersal and host-seeking ecology of "aggressive" bark beetles that use aggregation pheromones. Factors such as the beetle's amount of fat reserves, encountering of pheromone, and level of competition and host suitability determine whether a beetle joins resident beetles in colonizing a tree or is the first pioneer to attack.

Figure 6, a scheme is presented for dispersal flight under various conditions and circumstances. An individual would undertake a pioneer strategy, in my view, only if no pheromone were encountered during the dispersal, or after leaving unsuitable colonization areas, so that finally fat reserves became low (cf. Figure 5.6 in Byers, 1995). In this desperate state, the beetle would attempt to bore into any tree and, if lucky, would find a tree of low resistance (Figure 6). Thus, smaller beetles, those that suffered severe larval competition, or those that have used up their fat reserves in flight, regardless of size, are hypothesized to be the pioneers.

There are still many questions regarding the behavior of bark beetles during the dispersal and host selection period. For example, for how long do beetles fly in nature, how random are the paths, and how often do they land? There is a large body of literature on observations but few experiments from which conclusions can be drawn. The question as to what the flight paths of beetles might look like was investigated in the simulations that varied combinations of the EAR and AMT stepwise. The data for *I. typographus* [unflown in Duelli et al. (1997) and

Zolubas and Byers (1995)] were best fit if the beetles had a straight flight path after release. This is in accordance with the theory that newly emerged beetles would have fat reserves and tend initially to ignore pheromone and hosts. The data for flown *I. typographus* (Duelli et al., 1997), however, could not be fit by any combination of EAR–AMT. The method also focused on a combination of EAR–AMT for *T. lineatum*, where a quite small EAR of 0.2 m was needed with a winding AMT path of 36° to predict the recapture rates by Salom and McLean (1989) on the three trap rings of 5, 25, and 100 m radii. Although the flight time of beetles is unknown, this may not affect the recapture rates significantly because most beetles disperse outward and do not return (a 10-min flight gave similar results to the hour-long flight). One trap ring allows too many possible EAR–AMT pairs to be of any use in predicting flight paths, although some degree of circuitous path is indicated. In any case, simulations reveal that catch per trap ring in relation to radial distance can be influenced significantly by the beetle's AMT, which has not been measured directly in the field.

The equation and simulation models useful for analyzing other systems of trap rings can be obtained as a compiled program for IBM-compatible personal computers by downloading the software (RINGTRAP.ZIP) from the internet (http://www.vsv.slu.se/johnb/software.htm).

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CHEMICAL DEFENSE IN LARVAL TORTOISE BEETLES: ESSENTIAL OIL COMPOSITION OF FECAL SHIELDS OF *Eurypedus nigrosignata* AND FOLIAGE OF ITS HOST PLANT, *Cordia curassavica*

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Abstract—Larvae of the tortoise beetle *Eurypedus nigrosignata* construct fecal shields using cast skins and fecal strands. Survival of larvae with intact shields was higher in the field than for larvae with shields removed. In the laboratory, *E. nigrosignata* feculae had a deterrent effect on feeding in the ant *Myrmica rubra* as did an extract of the host plant, *Cordia curassavica*. Three chemical types were identified in the host-plant foliage and were named β-terpinene, α-pinene, and sabinene, depending on their mono- and sesquiterpene composition. This is the first report of lower terpenes (essential oils) in foliage of *Cordia*. Fecal shields of *E. nigrosignata* displayed the same terpene pattern as larval host-plant leaves. The absolute concentration of mono- and sesquiterpenes in the dorsal fecal shield depended on the plant chemical type and tended to decrease with larval age. No oxidation or detoxification products of ingested terpenes were detected in the larval fecula, indicating that the chemical composition of the larval fecal shield is influenced primarily by the host-plant secondary chemistry.

Key Words—*Eurypedus nigrosignata*, tortoise beetle, Chrysomelidae, *Cordia curassavica*, Boraginaceae, lower terpenes, essential oil, chemical type, chemotype, fecal shield, chemical defense.

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INTRODUCTION

Adult tortoise beetles (Cassidoid Hispinae, sensu Reid, 1995) are usually recognized by their extended elytra and pronotum. Larvae possess an equally striking morphological feature, a dorsal shield composed of molted skins attached to a forked appendage on the eighth segment (cf. Stehr, 1991, p. 569). Feculae are added to the structure, which is normally kept above the dorsum. The size and shape of the shield vary considerably among species (Muir and Sharp, 1904; Fiebrig, 1910; Engel, 1935; Gressit, 1952; Buzzi, 1988; Windsor et al., 1992). Different names are given to the fecal shields: larval clothing (Muir and Sharp, 1904), Kotanhang (trans. fecal appendage; Fiebrig, 1910), fecal masks (Engel, 1935), fecal shield (Eisner et al., 1967), faecal pad (Hawkeswood, 1982), and exuvio-faecal annex (Buzzi, 1988).

Various functions have been attributed to these structures (Fiebrig, 1910; Engel, 1935; Eisner et al., 1967; Balsbaugh, 1988; Olmstead and Denno, 1993; Olmstead, 1996). The effectiveness of the cassidine fecal shield in deterring ants and other predators has been demonstrated in the laboratory and in the field (Eisner et al., 1967; Olmstead and Denno, 1993; Gómez, 1997). The source and composition of repellent or deterrent properties in the larval fecal shields of Cassidinae remain largely uninvestigated (Pasteels et al., 1988; Blum, 1994; Olmstead, 1994, 1996). Morton and Vencl (1998) recently showed that larvae of the Criocerinae modified and incorporated plant noxious substances into their dorsal fecal clumps which were effective chemical barriers against ants. Still, one important question is whether recycling host-plant secondary compounds into the fecal shield of larvae is a general defensive strategy in the Cassidinae that may have contributed to the remarkable radiation of these beetles.

We selected the cassidine *Eurypedus nigrosignata* Boh. found exclusively on *Cordia curassavica* (Jacq.) Roem. & Schult. (Boraginaceae) in Central Panama to examine these questions. The pantropical genus *Cordia* L. is the largest genus in the Boraginaceae (Miller, 1988). Twenty-nine species are found in a wide range of habitats in the Republic of Panama; those in dry habitats are important components of the vegetation. *Cordia curassavica*, a deciduous shrub, 2–4 m tall, is common along roadsides from sea level to 2000 m. Six species of *Cordia* support 11 cassidine species in Central Panama (Windsor et al., 1992). The secondary chemistry of three Panamanian *Cordia* species has been reported. Ant-repellent triterpenoids were isolated from foliage of *C. alliodora* (Ruíz & Pav.) Oken (Chen et al., 1983), and phenolic terpenoids (Stevens et al., 1973; Stevens and Jurd, 1976) and a number of terpenoidal quinones were identified in its heartwood (Stevens et al., 1973; Moir and Thomson, 1973; Manners and Jurd, 1977). Allantoin, a urea-derivative, was isolated from *C. micrantha* Sw. (Lopez Vargas, 1982, as *C. collococca* var. *micrantha*). Gentisic acid was obtained from leaf tissue of *C. sebestena* L. from Trinidad (Griffiths, 1959). Additionally, lipo-

philic extracts of dried *C. curassavica* leaves were weakly active against the fungus *Cladosporium cucumerinum* (Cepleanu et al., 1994) and tumor cells (Chapuis et al., 1988) but were insufficient to kill crustaceans, mosquito larvae, or mollusks (Cepleanu et al., 1994).

Here, we report the effectiveness of the larval shield in the field; assay both larval fecula and plant secondary compounds in a feeding deterrent experiment with a laboratory predator; and isolate, characterize, and quantify secondary compounds in the foliage of *C. curassavica* as well as host-plant derived substances found in the fecal shields of *E. nigrosignata.*

METHODS AND MATERIALS

Insects. E. nigrosignata larvae were collected in Chepo (9°10′N/79°05′W) at the beginning of the rainy season (May–July) in each of three years (1994–1996). Larval broods with branches of their host plants were transported to the laboratory in separate plastic bags (Zip-Lock). Each larva was weighed, the fecal shield removed with forceps, and larva and shield weighed separately. Individual shields from the same broods were pooled as batches of 40 mg and then extracted. Shields from first instars usually were few and too small to reach this weight. Their extraction was carried out with the material available, usually no less than 20 mg.

Lower Terpenes in Larval Bodies. Six larvae with shields removed were kept in Petri dishes with moist filter paper and deprived of food for two days. Larvae were freeze-dried and macerated (30.3 mg) three times in 10 ml petrol ether at 40–60°C. Extracts were pooled and the solvent removed by rotary evaporation under reduced pressure. The residue was analyzed by GC-MS.

Plants. Foliage of *C. curassavica* was collected in Central Panama (9°10′N/79°05′W). Branches were transported to the laboratory in separate plastic bags (Zip-Lock). Young leaves were excised and extracted immediately.

For comparisons between leaves of different ages from both field-collected and greenhouse plants, bulk collection was carried out. Leaves were sorted by age class, dried at room temperature, packed in plastic bags, and kept in a dry, cool place until analysis. Although a similar storage procedure has been shown to result in loss of volatiles (Ross and ElSohly, 1996), it proved adequate for our purpose.

Cultivated shrubs were 1 year old when used for chemical extraction and for maintaining a population of *E. nigrosignata.* Seeds used for cultivation were collected in Central Panama in 1994 and sown in the greenhouse of the Pharmaceutical Biology Institute in Braunschweig, Germany. The light regime in the greenhouse was a 14-hr day from October to April. For both collections, newly expanded leaves on nodes 1 and 2 were designated "young leaves"; leaves on

nodes 3–5, "mid-aged leaves"; and leaves on nodes >5 "old leaves." Statistical comparisons between leaves of different age classes ($N = 5$) were carried out as indicated below.

Because this was the first report of lower terpenes occurring in leaves of a *Cordia* species, three other species, also eaten by Cassidinae, were analyzed: *C. linnaei* (El Llano-Cartí), *C. spinescens* L. (Old Gamboa Road), and *C. alliodora* (Pipeline Road). Young foliage was extracted as described below. Foliage at all sites was collected at the same time of day.

Field Experiment. Each of 47 pairs of equal-sized larvae were placed on individual *C. curassavica* plants. Each pair consisted of a larva with an intact shield and another with the shield removed. Six pairs were larvae in the fourth stadium, the remainder between first and third stadium. Larvae were counted again after 24 hr. Results were evaluated statistically as indicated below. Larval disappearance was assumed to be due to predation. Isolated shrubs were chosen to eliminate the possibility of movement among plants.

Feeding Deterrent Experiment. A lower terpenoid extract from foliage of field-collected plants was obtained by steam distillation. The beetle population, raised on greenhouse-cultivated plants, originated from egg masses and gravid females brought from Panama in June 1995. Larvae and the food plant were transported to Brussels in October 1995. *Myrmica rubra* reared at the Institute of Animal and Cellular Biology, College of Natural Sciences, Free University of Brussels, Belgium, was used as a predator. Frozen larvae of the house fly, *Musca domestica*, were used as a food reward in the feeding experiments.

Thirty foragers of *M. rubra* were isolated in a closed Petri dish (150 × 15 mm) and starved overnight. Drinking water was removed 1 hr before running the experiment. A binary divider was placed inside the Petri dish (Pasteels et al., 1986). Every minute for 10 min and every 5 min for another 10 minutes, ants visiting the feeding sites were counted, e.g., ants drinking or cleaning themselves immediately around each site of the device. Ant visitation percentage at a particular time was calculated by dividing the number of ants at a site by the total number of ants (sum of both sites). For each replication, a different group of 30 foragers was used. All experiments were carried out at 23–25°C and 60% humidity.

M. rubra was presented with a choice between food reward mixed with *E. nigrosignata* feculae and food reward without feculae. Under the stereomicroscope, fecal strands and cast skins were separated from 15 dorsal shields (103.2 mg) freshly removed from larvae between the second and third stadium. Fecal filaments (58.3 mg) were crushed with 15 frozen house fly larvae (83.2 mg/larva) in 0.7 ml distilled water. The suspension was collected in a glass vial, and 50 μl was placed in a paraffin hole (treated sited) in the test device. The same volume of food reward prepared without fecula was placed in the other hole. This hole became the control site. The concentration before adding water

was 0.14 μg lower terpenoid/mg food mixture. The position of the test device in the Petri dish was inverted between replicates. Eight replicates were run and evaluated statistically. The concentration of lower terpenoids in the fecal shields of *E. nigrosignata* raised in the greenhouse was 0.57 μg lower terpenoid/mg larva (fresh weight basis) and carried the β-terpinene type.

M. rubra was also presented with a choice between a food reward mixed with an extract of lower terpenoids from field-collected *C. curassavica* and one without the extract. The food reward was prepared by crushing 15 frozen house fly larvae in 0.7 ml distilled water. After collecting the suspension in a glass vial, 50 μl were placed in each paraffin hole of the test device. Using a 10-μl Hamilton syringe, the extract of lower terpenoids (5 μl) was added carefully to a hole containing the food reward (treated site). Hexane (5 μl) was added to the other hole (control site). After 10 min at room temperature to allow the solvent to evaporate, the device was placed inside the Petri dish with ants. Five microliters of the plant extract corresponded to 10.2 μg essential oil. After addition to 50 μl food reward, the final concentration was 0.16 μg lower terpenoid/mg food mixture. The position of the test device in the Petri dish was inverted between replicates. Seven replicates were carried out and evaluated statistically. The field-collected foliage carried the α-pinene type.

Taxonomic Identification. Cassidinae and their host plants were identified by D. Windsor, Smithsonian Tropical Research Institute (STRI), by comparison to type material in the British Natural History Museum. Voucher specimens are deposited in the insect collection and the herbarium of the STRI Research and Conference Center.

Compound Extraction. The method described by Pare (1994) was modified to extract lower terpenoids quantitatively from small quantities of plant and insect tissues. In a typical trial, 10–100 mg of plant or insect material was weighed into 2 ml-Twist Cap vials (conical, polypropylene, screw caps with O-ring seal) containing 2 glass beads (4–mm diam.). After addition of 0.75 ml hexane containing 50 μg/ml of *n*-octadecane and *n*-hexadecanol as internal standards, the tightly closed vial was vortexed at medium speed for 2 min (or until the material was crushed). Vials then were transferred to a vial holder placed on a 400-ml glass beaker containing 50 ml water. The beaker with the samples was heated in a microwave oven at full power (750–800 W) for 1 min. Samples were allowed to cool at room temperature, transferred to 1.5-ml Eppendorf vials, and centrifuged. The clear supernatant was kept in tightly sealed 2.0-ml glass vials (screw cap with Teflon lining) at −18°C until GC and GC-MS were carried out. The method is applicable to fresh or dried plant or insect samples in amounts of 10–100 mg. Glass beads were unnecessary when fresh plant material was extracted, because glandular trichomes on the leaf surface were destroyed by hot hexane (personal observation).

Steam distillation was performed in a steam-distillation apparatus as de-

scribed by the German Pharmacopoeia (DAB10, 1991). Dried plant material (1 g) was placed in a flask, 200 ml distilled water were added, and the distillation carried out for 5 hr. The distillate was collected in 0.75 ml hexane. *n*-Octadecane was added to reach a final concentration of 20 μg/mg. Plant specimens for the laboratory experiment also were extracted in this fashion.

Capillary Gas Chromatography (GC). Separation and quantification of lower terpenes were carried out in a Carlo Erba Mega gas chromatograph equipped with an on-column capillary injector and a flame ionization detector (FID). A fused-silica capillary column, 30 m × 0.32 mm with 0.25-μm film thickness of bonded methyl silicone (DB-1, J&W Lt., Folsom, California) was applied. GC conditions were the following: detector temperature, 300°C; temperature program, 70–250°C with hexane at a rate of 15°C/min; carrier gas, helium (120 kPa). Quantification of the FID signals was achieved with a Shimadzu integrator C-R6A Chromatopac, with *n*-octadecane and *n*-hexadecanol as internal standards.

Gas Chromatography–Mass Spectrometry (GC-MS). Separation and identification of lower terpenoids were accomplished in a Carlo Erba Mega gas chromatographer equipped with a split–splitless injector directly coupled to a Finnigan Mat 4515 quadrupolspectrometer. A fused-silica capillary column was used for the GC separation. GC conditions were: injector temperature, 250°C; temperature program 70–250°C at a rate of 6°C/min; split injection, split ratio, 1 : 20; carrier gas helium (50 kPa). Retention indices (RIs) were determined by coinjecting *n*-alkane standards (C_9–C_{32}) (Kováts, 1965). EI mass spectra were recorded at 50 eV. The identification of individual compounds was achieved by comparison of RIs, molecular ion [M^+], and fragmentation patterns with the MS libraries of the Chemistry Department of the Braunschweig Technological University and the perfume company Dragoco in Holzminden, Germany.

Scanning Electron Microscopy. The leaf surface of freshly collected young leaves of *C. curassavica* was viewed directly using back-scattered electrons at low vacuum and photographed in a scanning electron microscopy Jeol model 5300LV SEM.

Statistical Procedures. Values were examined for normality and homoscedasticity (Levine's test). Data were natural log transformed when necessary. A two-factorial ANOVA, with site and leaf-age class as treatments, was used to compare results from plants grown in the greenhouse and those collected in the field. Means of age-class groups were compared by a Tukey *t* test. Chemical types of fecal shields were compared by a two-tail *t* test. Larval weight percentages were arc-sin transformed, examined for normality and homoscedasticity (F test), and compared by a single-factorial ANOVA with weight as treatment. Means of instar weight percentages were compared by a Tukey *t* test. Values were back-transformed and their confidence limits calculated using critical values based on the Sidak's multiplicative inequality. The larval survival field experiment was eval-

uated by categorial analysis. Results from feeding experiments in the laboratory using *M. rubra* were analyzed by the Wilcoxon signed-ranks test (Pasteels et al., 1986). All procedures were carried out using Systat (Systat, 1992).

RESULTS

Effectiveness of Larval Fecal Shields. E. nigrosignata larvae are usually found on the upper surface of young leaves of their host plant. Larvae normally emerge from eggs on the abaxial surface of the natal leaf and climb upward to the exposed apical leaves where they begin to feed (Figure 1). Larvae of all stadia rapidly rotate their abdomens and dorsal shields toward sources of physical disturbance. The protective role of the shield was evaluated in a field experiment (Figure 2). Survival of larvae with intact shields was higher than that of larvae without shields (G test: $G = 4.663$, $N = 94$; $P < 0.05$). During the course of the experiment larvae that had their shields removed began to construct new shields by using fresh fecal filaments, possibly increasing their survival.

Feeding Deterrence. The feeding behavior of *M. rubra* was assessed by presentation of *E. nigrosignata* feculae mixed with a food reward. Overall, feculae had a negative effect on ant feeding behavior (Figure 3A). During the first 2 min, the number of ants visiting the two feeding sites did not differ significantly. After this lapse, and for 20 min, the feeding activity at the treated site remained significantly lower than that at the control site. During the experiment, the percentage of ant visitation to the treated site oscillated between 32% and 42%. The postingestive effect was not evaluated.

Another choice experiment was conducted with an extract of lower terpenoids obtained from *C. curassavica* by steam distillation and mixed with a food reward. The plant extract had a negative effect on the feeding behavior of *M. rubra* (Figure 3B) during all except the first minute of the experiment. The percentage of ants visiting the treated site ranged between 8% and 16% during the total observation time. Ants approached and often backed away quickly without tasting the bait; only a small number were observed to feed.

Lower Terpenoids of Cordia *Species.* Concentrations of lower terpenoids (on dry weight basis) in the foliage of *C. curassavica* ranged from 1.2 to 2.3%, was 0.7% in *C. spinescens*, while *C. alliodora* and *C. linnaei* were devoid of these compounds. Lower terpenoids of *C. curassavica* are likely to be localized in glandular trichomes on the adaxial and abaxial surfaces (Figure 4A and B); nonglandular hairs on the abaxial surface were longer than those on the adaxial surface. Glandular hairs of the same basic form were found on leaves of *C. spinescens* (not shown). *Cordia alliodora* and *C. linnaei* were devoid of glandular trichomes.

Three chemical types in *C. curassavica* were distinguishable by their mono-

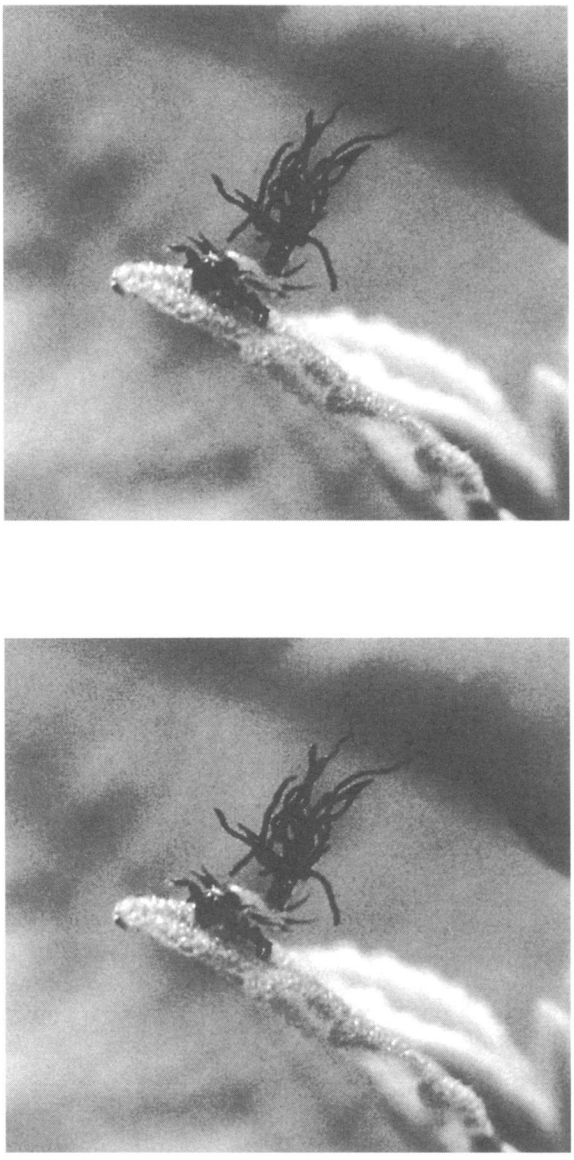

FIG. 1. *E. nigrosignata* larvae on their host plant *C. curassavica*. Larvae (2nd stadium) feeding on the upper surface of a young leaf. The fecal shields are constructed of molted skins and fecal strands. A peristaltic anus delivers single fecal filaments outwards, which are then successively attached to the exuvial base (possibly by a gluelike secretion).

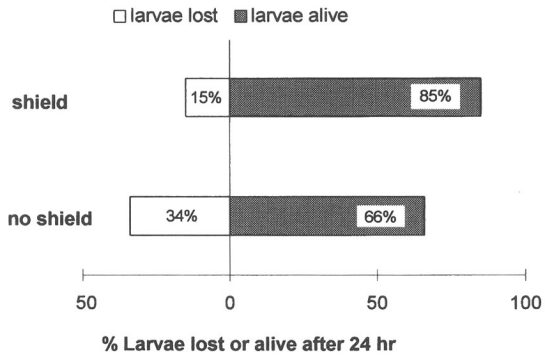

FIG. 2. Percentage of *E. nigrosignata* larvae that survived in the field after 24 hr. $P < 0.05$.

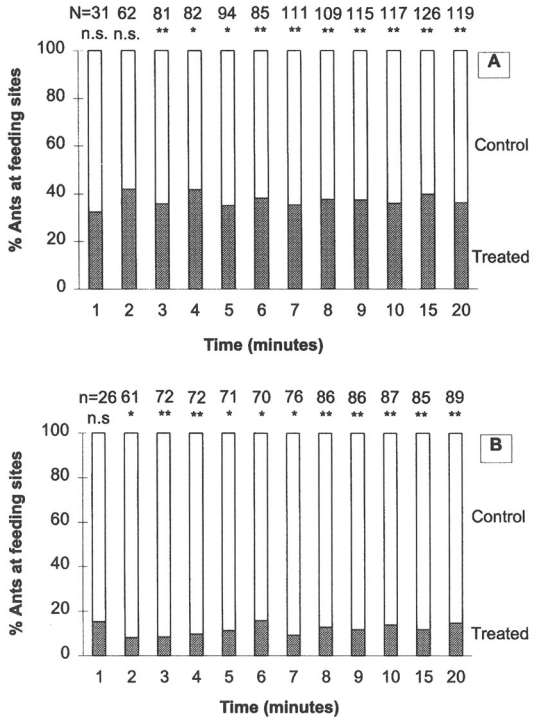

FIG. 3. Feeding activity of *M. rubra* in a binary choice experiment using (A) fecula of *E. nigrosignata* and (B) an extract of lower terpenoids from wild specimens of *C. curassavica*. The dark area is the treated site; the clear area is the control site (Wilcoxon signed-ranks test: $^{*}P < 0.05$, $^{**}P < 0.02$, and n.s., not significant).

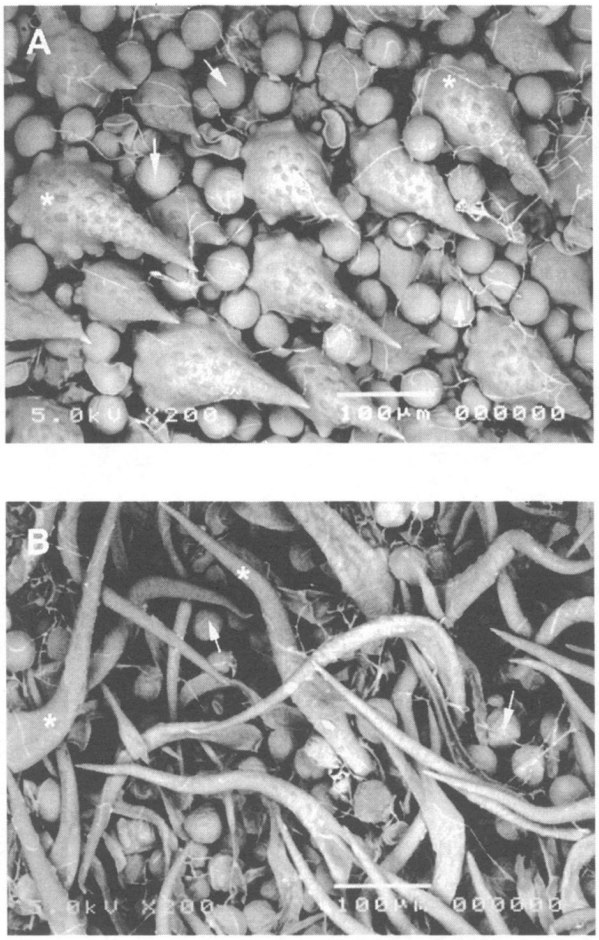

FIG. 4. Scanning electron microscopic (SEM) magnification of (A) the upper and (B) lower surface of very young leaves of *C. curassavica*, showing glandular trichomes (round, with arrows) intermingled with nonglandular trichomes (long, with asterisks).

terpene composition (Table 1). Each type was grouped by terpenoid class, i.e., monoterpenes and sesquiterpenes, and the relative mean concentration (% in Table 1) was calculated for each type, terpene class, and individual terpenoid. Overall, plants showed high variability of lower terpenoids, as indicated by the standard deviation of values. Individual plants within a chemical type contained single terpenes that were absent in other plants.

The "β-terpinene type" contained 12 monoterpenes and 22 sesquiterpenes.

TABLE 1. CHEMICAL TYPES OF *C. curassavica* FOLIAGE AND FECAL SHIELDS OF *E. nigrosignata* REMOVED FROM LARVAE FEEDING ON LEAVES WITH β-TERPINENE AND α-PINENE TYPES, RESPECTIVELY[a]

			β-Terpinene type				α-Pinene type				Sabinene type	
			C. curassavica (*N* = 3)		*E. nigrosignata* (*N* = 6)		*C. curassavica* (*N* = 4)		*E. nigrosignata* (*N* = 12)		*C. curassavica* (*N* = 2)	
	RI	$[M]^+$	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%
Monoterpenes												
β-Terpinene	920	136	3.96 ± 1.00	32	3.28 ± 1.95	33	tr.				tr.	
α-Pinene	927	136	0.80 ± 0.25	6	0.52 ± 0.39	5	7.50 ± 1.35	39	1.74 ± 1.07	39	0.85 ± 0.03	4
Camphene	935	136	1.06 ± 0.39	9	0.69 ± 0.51	7	0.12 ± 0.04	1	tr.			
Sabinene	960	136	tr.		tr.		0.39 ± 0.17	3	0.08 ± 0.05	2	14.30 ± 0.31	61
β-Pinene	970	136	0.24 ± 0.25	2	tr.		3.80 ± 1.22	20	0.63 ± 0.37	14	0.40 ± 0.01	3
Myrcene	980	136	tr.		tr.		0.47 ± 0.19	2	0.05 ± 0.04	1		
Cineol	1018	154	tr.		tr.		tr.		0.04 ± 0.03	1	tr.	
β-Phellandrene	1018	136	0.18 ± 0.10	1	0.16 ± 0.10	2	0.58 ± 0.16	3	0.08 ± 0.09	2	0.52 ± 0.00	2
Limonene	1020	136	tr.		tr.		0.11 ± 0.09	1	0.07 ± 0.03	2		
trans-β-Ocimene	1035	136	0.16 ± 0.05	1	0.09 ± 0.04	1	0.19 ± 0.08	1	0.05 ± 0.03	1		
Bornyl acetate	1265	196	tr.				tr.		0.03 ± 0.03	1		
Unknown (acetate)	1323	154	tr.		tr.		tr.		tr.			
Monoterpene total			6.54 ± 1.90	53	4.89 ± 3.00	50	13.58 ± 2.88	71	2.79 ± 1.64	62	16.20 ± 1.04	70
Sesquiterpenes												
δ-Elemene	1330	204	0.12 ± 0.04	1	0.10 ± 0.03	1	0.20 ± 0.08	1	0.09 ± 0.04	2	0.22 ± 0.06	1
α-Copaene	1367	204									0.32 ± 0.03	1
β-Copaene	1380	204	0.15 ± 0.05	1	0.15 ± 0.12	1	0.13 ± 0.07	1	0.06 ± 0.02	1		
β-Elemene	1382	204	0.09 ± 0.03	1	0.08 ± 0.07	1	tr.		tr.			
Elemane type[b]	1395	204	tr.		0.06 ± 0.01	1	0.08 ± 0.04	1	0.04 ± 0.02	1		
Guaiane type[b]	1403	204	0.62 ± 0.87	5	0.34 ± 0.66	5						
Caryophyllene	1405	204	1.50 ± 0.58	12	1.31 ± 0.71	13	2.86 ± 0.84	15	0.71 ± 0.53	16	1.33 ± 0.30	6
γ-Elemene	1424	220					tr.		tr.			
6,9-Guajadiene	1430	204	tr.		0.06 ± 0.03	1	tr.		tr.			

TABLE 1. Continued

			β-Terpinene type				α-Pinene type				Sabinene type	
			C. curassavica (*N* = 3)		*E. nigrosignata* (*N* = 6)		*C. curassavica* (*N* = 4)		*E. nigrosignata* (*N* = 12)		*C. curassavica* (*N* = 2)	
	RI	$[M]^+$	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%
Sesquiterpenes (Continued)												
α-Humulene	1440	204	0.27 ± 0.07	2	0.24 ± 0.12	2	0.42 ± 0.12	2	0.10 ± 0.06	2	2.17 ± 0.39	9
Germacrene D	1467	204	1.65 ± 0.74	9	0.59 ± 0.71	6	0.38 ± 0.21	2	0.11 ± 0.11	2	tr.	
γ-Selinene	1470	204	0.21 ± 0.30	2	0.24 ± 0.31	2						
Selinene isomer[b]	1470	204	0.18 ± 0.26	2	0.22 ± 0.31	2						
Bicyclogermacrene	1478	204									tr.	
δ-Selinene	1480	204	0.13 ± 0.19	1	0.22 ± 0.30	2						
Selinane type[b]	1484	204	tr.		tr.						0.84 ± 0.15	4
α-Amorphene	1488	204	0.14 ± 0.20	1	0.07 ± 0.12	1	0.49 ± 0.15	2	0.18 ± 0.09	4	0.26 ± 0.03	1
Germacrane type[b]	1490	222									0.35 ± 0.01	2
Selinane type[b]	1498	222	tr.		tr.							
(3,11?)-Selinadiene[b]	1501	204	0.10 ± 0.15	1	0.10 ± 0.18	1						
δ-Cadinene	1507	204	0.08 ± 0.10	1	0.07 ± 0.11	1	0.37 ± 0.10	2	0.07 ± 0.02	1	0.40 ± 0.09	2
Germacrane type[b]	1540	222	0.23 ± 0.19	2	0.14 ± 0.17	1					0.30 ± 0.06	1
Elemol[b]	1550	222	0.25 ± 0.35	2	0.29 ± 0.37	3	0.33 ± 0.18	2	0.11 ± 0.1	2		
Unknown	1575	222	0.24 ± 0.30	2	0.22 ± 0.28	2	tr.		0.04 ± 0.06	1		
Germacren-1,6-dien-5-ol[b]	1594	222	0.14 ± 0.13	1	0.07 ± 0.05	1						
δ-Cadinol	1620	222	tr.		0.07 ± 0.11	1	tr.		0.03 ± 0.05	1	0.34 ± 0.01	2
β-Eudesmol	1622	204					tr.		tr.		0.25 ± 0.00	1
Unknown[c]	1738	236					tr.		tr.			
Sesquiterpene total			5.73 ± 1.84	47	4.93 ± 2.42	50	5.56 ± 1.55	29	1.68 ± 0.70	38	7.05 ± 1.09	30
Total			12.27 ± 3.12		9.83 ± 4.59		19.14 ± 4.00		4.47 ± 2.08		23.34 ± 2.22	

[a]RI, retention index; $[M]^+$, molecular ion; SD, standard deviation; tr., traces.
[b]Terpenoid type or isomer.
[c]Dioxygenated sesquiterpene.

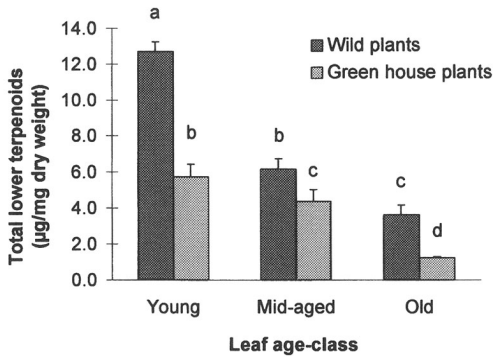

FIG. 5. Total mean concentration ($N = 5$) of lower terpenes in *C. curassavica* leaves of increasing age. Leaf-age within a site, and plants among sites are compared; letters on bars indicate significant differences. $P < 0.001$.

Major monoterpenes were β-terpinene, α-pinene, and camphene, representing 89% of the monoterpene fraction. Among the sesquiterpenes, a nonidentified guaiane type structure (RI 1403), caryophyllene, and germacrene D accounted for almost 55% of the sesquiterpene fraction. The "α-pinene type" was composed of 12 monoterpenes and 16 sesquiterpenes. α-Pinene and β-pinene made up 83% of the monoterpene fraction. Caryophyllene was the main sesquiterpene (51% of the sesquiterpene fraction). The "sabinene type" was characterized by sabinene (87% of the monoterpene portion). Caryophyllene and α-humulene were the major sesquiterpenes comprising together 15% of all terpenoids and 50% of sesquiterpenes.

Young foliage from both wild and greenhouse plants carrying the β-terpinene type contained significantly higher amounts of terpenoids on a dry weight basis than did mature foliage (Figure 5); however, the total yield of lower terpenoids was significantly higher in field-collected plants (two-factorial ANOVA: $F = 23.92$; df 2,24; $P < 0.001$; Tukey t test: $N = 30$; $P < 0.05$).

Lower Terpenoids of Larval Fecal Shields. Fecal shields of *E. nigrosignata* larvae contained the same terpenoidal profile found in the foliage on which the larvae had fed, β-terpinene and α-pinene, respectively, and in similar relative amounts (Table 1). In addition, the total concentration of the β-terpinene type was significantly higher than that of the α-pinene type ($P < 0.03$, two-tailed t test, $N = 18$). The concentration of lower terpenoids in larval shields also tended to decline with larval age regardless of the chemical type (Table 2). The larval fecal shield weight, as a percentage of the total body weight, declined significantly (Table 2). Products of metabolism or detoxification, such as oxidized terpenoids, were not detected in the fecal shields. Similarly, plant-derived terpenoids were not found in body tissues of starved larvae.

TABLE 2. WEIGHTS OF THREE OF *E. nigrosignata* INSTARS, CONTRIBUTION OF RESPECTIVE FECAL SHIELDS TO TOTAL LARVAL WEIGHT AND THEIR LOWER TERPENOID CONCENT IN FECAL SHIELDS

	First stadium	Second stadium	Third stadium
Larval weight (mg, mean ± SD)[a]			
Total larvae	6.91 ± 3.29	19.01 ± 3.63	31.54 ± 4.01
Body (fecal shield removed)	4.77 ± 2.28	13.56 ± 2.89	23.24 ± 3.62
Fecal shield	2.15 ± 1.10	5.45 ± 1.26	8.30 ± 1.37
Fecal sheild			
Mean % of total larval weight)[b]	31.0	28.7	26.5
Confidence interval	28.9–33.1	26.8–30.6	24.2–28.9
Fecal shields lower terpenoid content (μg/mg ± SD dry wt)			
α-Pinene host plant[c]	6.72 ± 2.10	4.87 ± 1.80	3.37 ± 0.90
β-Terpinene host plant[d]	12.82	10.60 ± 2.87	6.75

[a] N = 44 (1st stadium); N = 40 (2nd stadium); N = 18 (3rd stadium).
[b] Back-transformed means and confidence intervals; $P < 0.01$, ANOVA yielded a significant difference between groups; $P < 0.05$, Tukey t test showed a significant difference between 1st and 3rd instars.
[c] N = 2 (1st stadium); N = 5 (2nd stadium); N = 5 (3rd stadium).
[d] N = 1 (1st stadium); N = 4 (2nd stadium); N = 1 (3rd stadium).

DISCUSSION

Young leaves of *C. curassavica* contained higher concentrations of lower terpenoids than did mature foliage, in both the field and the greenhouse. This finding agrees with studies on other plants containing lower terpenoids (Langenheim, 1994 and references therein; Ohmart, 1996). In the taxonomically related Lamiaceae, terpene biosynthesis is more rapid at the early stages of leaf development (Croteau et al., 1981; Gershenzon, 1994). In mature leaves, the number of oil glands and total oil production becomes limited (Croteau et al., 1981), resulting in an overall decrease in the lower terpenoid tissue concentration. Similarly, plants growing under greenhouse conditions yield lower amounts of lower terpenoids (Fahlen et al., 1997).

Unlike several other neonate cassidine larvae, which begin to feed on their natal leaf upon hatching (Engel, 1935; Hawkeswood, 1982; Windsor, 1987), newly emerged *E. nigrosignata* larvae frequently move upwards to more apical foliage. Their first feeding site is normally the upper surface of the youngest, most tender leaves. This behavior may be a response to larval nutritional demands (Morrow and Fox, 1980; Scriber and Slansky, 1981; Coley, 1983; Ohmart et al., 1985) and/or to plant defensive compounds (Larsson et al., 1986; Larsson and Ohmart, 1988; Björkman and Larsson, 1991; Selman, 1994; Coley and Kursar, 1996). Young foliage of *C. curassavica* has a higher content of lower

terpenoids, which, in turn, is voided into the dorsal shields by larvae. Young cassidine larvae are more susceptible to predation than older ones (Olmstead and Denno, 1993; Olmstead, 1996; Gómez, 1997). Therefore, larvae feeding on younger leaves when they emerge should become better and more quickly protected than larvae feeding on older leaves. Moreover, feeding that occurs primarily on the adaxial side of young leaves may allow larvae to reach glandular trichomes more easily than when feeding on the abaxial surface, where longer nonglandular trichomes are more abundant and may make access more difficult.

Fecal shields of older larvae contained lower terpenoid concentrations than did shields of younger instars. This decrease may reflect natural feeding habits. For example, late instars tended to move to mature foliage. Lower terpenes were not found in body tissues of starved larvae, thus indicating that larvae did not sequester these compounds. Monoterpenes from *Eucalyptus* trees also were absent from the body tissue of paropsine larvae (Chrysomelinae) (Morrow and Fox, 1980; Ohmart and Larsson, 1989), but appeared unchanged in the fecula (Morrow and Fox, 1980).

Larval voiding of unaltered plant compounds could be advantageous from both a metabolic and energetic standpoint (Eisner et al., 1974; Bowers, 1990; Blum, 1994), especially if detoxification processes, such as oxidation and conjugation, are minimal and the compounds are still active against enemies. The fecal shields of *E. nigrosignata* contained the same terpenoidal composition of the foliage they ingested. Moreover, larval fecal shields with the β-terpinene type contained higher amounts of lower terpenes than did shields carrying the α-pinene type. This difference was related to the sesquiterpene fraction. Indeed, foliage with this type contained a number of sesquiterpenes that were absent or undetectable in plants with other chemical types, thereby showing the influence of a particular host plant on the chemical composition of the fecal shield.

It is conceivable that a shield containing a higher content or a greater diversity of noxious substances is beneficial, as has been suggested for lower terpenoids in plants (Pimentel and Belloti, 1976; Berenbaum, 1985; Bryant et al., 1992) or for other compounds in insect defensive secretions (Pasteels et al., 1996; Schoeder et al., 1998). Monoterpenes disrupt a number of physiological functions in insects (Brattsten, 1983; Bowers, 1990), and feeding on plants with higher amounts of lower terpenes could impair larval performance (Larsson et al., 1986; Björkman and Larsson, 1991; Zou and Cates, 1997, but see Morrow and Fox, 1980 for a different outcome) and development time (Zou and Cates, 1997). It still is unclear whether or not *E. nigrosignata* preferentially feeds on plants with a particular chemical type that may affect its performance and growth or influence the effectiveness of its shields against particular enemies.

If plant-derived lower terpenoids in the dorsal fecal shield are involved in the chemical defense of *E. nigrosignata* larvae, it is important to determine whether they function on contact (deterrent) or at a distance (repellent). Volatile

monoterpenes are expected to be more repellent than sesquiterpenes, since they volatilize faster and may be released in higher amounts when larvae swat their shields, for instance, upon approaching enemies (Hawkeswood, 1982; Olmstead, 1994; Gómez, 1997). The allomonal effect of lower terpenes in larval shields may be dose dependent (Agelopoulus et al., 1995; Alborn et al., 1995) and/or release-dependent (Agelopoulus et al., 1995).

While insect feculae and the plant essential oil had a negative effect on ant feeding behavior, the responses were different. Ants were repelled rather than deterred during the experiment with the plant extract. Ants retreated after initially approaching the treated drops. When feculae were used, ants tasted the test drops first, then ate or left. The difference in feeding behavior might be explained partially by the characteristics of the food reward. The plant extract was applied over a drop of the food, whereas the fecula was crushed with the food. Substances on the surface may vaporize faster than those embedded in a matrix. On the other hand, a long-lasting repellent effect (over 20 min, personal observation) could result from the lipophilic nature of the food. Fats released from the house fly larval body may have provided a good matrix to dissolve these apolar substances, especially on the surface of the test drop. A dose dependency may also have occurred with fecula. The final concentration of lower terpenoids in the diet was four times lower than the concentration determined per larvae raised in the greenhouse. It decreased more after addition of water during the diet preparation, and may have caused a less pronounced response of the laboratory predator. Major monoterpenes of the chemical types identified in our study have been shown to bring about differential responses in insects (Nehlin et al., 1996). Further experiments which use extracts from dorsal shields of *E. nigrosignata* larvae against natural enemies would help evaluate the chemical protection by lower terpenoids.

Lower terpenoids are known to be active in a number of prey–predator interactions. α-Pinene and β-pinene, major monoterpenes of the α-pinene-type in our study, occur in defensive discharges of *Neodiprion sertifer* (Hymenoptera: Diprionidae), and they deter spiders and ants (Eisner et al., 1974), *N. sertifer* sequesters these compounds from its host plant *Pinus sylvestris*. α-Pinene is the main monoterpene in the defensive discharge of *Nasutitermes princeps* (Termitidae) (Everaerts et al., 1990) and elicits alarm reactions of large workers (Roisin et al., 1990). α-Thujene, sabinene, myrcene, α-phellandrene, α-terpinene, terpinolene, and limonene, which are found in the larval fecal shields of *E. nigrosignata*, are minor monoterpenes in the defensive secretion of termites. The oral discharge of sawflies of the genus *Pseudoperga* (Pergidae) contains mono- and sesquiterpenes that are derived from *Eucalyptus* spp. and are effective in repelling mice, birds, and ants (Morrow et al., 1976).

Volatile defensive substances can be exploited by enemies for their benefit (Vet and Dicke, 1992). The role of volatile compounds emitted from insect fecula positively influenced the host-searching behavior of herbivore predators

(van Leerdam et al., 1985; Alborn et al., 1995; Agelopoulus et al., 1995). Olmstead (1994, 1996) has stressed the drawback of using waste materials containing volatiles as defensive agents due to their potential kairomonal effect. Yet, the larval fecal shield of *E. nigrosignata* contained highly volatile monoterpenes.

The field experiment indicates that dorsal shields contribute to the survival of *E. nigrosignata* larvae. However, the survival of nonshielded larvae was also considerable, perhaps due to their behavior in immediately beginning to construct new shields. Within 18 hr, a larva is able to build a new shield, although only made of fecal strands (personal observation). Larval size may also have positively influenced survival. Larger larvae of the Cassidinae, even without shields, had better survival than smaller ones (Olmstead and Denno, 1993; Gómez, 1997). In general, cassidine larvae suffer low survival rates in natural settings (Olmstead and Denno, 1993; Olmstead, 1996; Becker and Pires Freire, 1996; Gómez, 1997).

There are few studies addressing the chemical composition of larval fecal shields in the Chrysomelidae and the secondary chemistry of their host plants (Gómez, 1997; Morton and Vencl, 1998). Plant-derived substances in the fecal shield of Criocerinae, however, do protect larvae against natural enemies (Morton and Vencl, 1998). Like other Cassidinae, *E. nigrosignata* and its host plant *C. curassavica* is an excellent model to assess defensive attributes based on chemical compounds. From the phytocentric point of view, it is worthwhile to further examine the occurrence of chemical types and their significance. From the zoocentric point of view, feeding on particular chemical types may or not prove to be advantageous.

Studies of host specialization, herbivore plant-derived chemical defenses, their effectiveness, and target enemies will contribute to understanding the evolution of the dorsal shield as a conveying device for defensive substances and its role in the diversification of the tortoise beetles.

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INTRA- AND INTERSPECIFIC AGGREGATION RESPONSES OF *Locusta migratoria migratorioides* AND *Schistocerca gregaria* AND A COMPARISON OF THEIR PHEROMONE EMISSIONS

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Abstract—Gregarious nymphs of *Schistocerca gregaria* and *Locusta migratoria migratorioides* are often seen to march together in common bands in the field. In the present study, the intraspecific aggregation responses of nymphal and older stages of the two insects were compared with their interspecific responses. Unlike *S. gregaria*, *L. m. migratorioides* shows a lesser pattern of stage and sex differentiation in its aggregation pheromone biology. Thus, although fifth-instar nymphs did not respond significantly to the adult pheromone, adults responded significantly to the nymph pheromone. No cross-stage aggregative responses occur in *S. gregaria*. In the adults of *S. gregaria*, production of the pheromone is male-specific; in *L. m. migratorioides* both sexes induced a significant level of aggregation from conspecifics. Aggregation assays between corresponding stages of the two species showed stronger interaction between the nymphal stages than between the adults and account for the frequent occurrence of mixed hopper bands in the field. GC-EAD studies of volatiles of nymphal and adult stages by using antennal preparations from both species also showed significant interspecies reactivities. GC-MS analyses showed that of *S. gregaria* nymphal pheromone components (C_6 and C_8 to C_{10} straight-chained aldehydes and acids and the fecal phenols, guaiacol and phenol), the acids and phenols constitute the common components of nymphal stages. Phenylacetonitrile, the major component of *S. gregaria* adult

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aggregation pheromone, is present in *L. m. migratorioides* nymphal volatiles, albeit at a lower level. Unlike *S. gregaria* adult pheromone blend, which is made up wholly of benzene derivatives, the volatile emissions of *L. m. migratorioides* adults is dominated by aliphatic aldehydes and alcohols. The possible role of cross-aggregation effects in mutually facilitating phase transformation of the two species is discussed.

Key Words—*Schistocerca gregaria*, *Locusta migratoria migratorioides*, locust, aggregation, pheromone, aliphatic acids, aldehydes, alcohols, benzene derivatives.

INTRODUCTION

The African migratory locust *Locusta migratoria migratorioides* (R&F) and the desert locust *Schistocerca gregaria* (Forskäl) both show density-dependent phase polymorphism, transforming reversibly between two extreme phases, solitaria and gregaria, under favorable environmental conditions (Uvarov, 1966). Grouping behavior is a characteristic feature of their gregarious forms and is mediated by pheromones (Gillett et al., 1976; Fuzeau-Braesch et al., 1988; Obeng-Ofori et al., 1993, 1994a,b; Torto et al., 1994, 1996). The recession habitats of the two species overlap somewhat in the Sahelian belt (Steedman, 1988). Their gregarious nymphs are often seen to march together in common bands, suggesting an overlap in their communication systems (Johnston and Buxton, 1949; El-Bashir and Abdel-Rahman, 1991).

One attempt to compare the composition of volatiles associated with the two species suggested only quantitative differences, irrespective of the stage of the insects (Fuzeau-Braesch et al., 1988). However, the method used to trap the volatiles was inefficient (Obeng-Ofori et al., 1993), and subsequent studies revealed a much richer and more complex pheromone system for *S. gregaria* (Obeng-Ofori et al., 1994a,b; Torto et al., 1994, 1996). The latter studies showed clear stage- and sex-related differentiations in pheromone composition, production, and aggregation responses. Specifically, gregarious second- to fifth-instar stages of *S. gregaria* aggregated in response to a blend of compounds dominated by aldehydes, their corresponding acids, and fecal phenols associated with both nymphal sexes (Torto et al., 1996). The adult stages are indifferent to this blend but respond to a pheromone mixture of benzene derivatives produced by older and mature males (Torto et al., 1994).

The present study was undertaken with the purpose of comparing the intraspecific aggregation responses of nymphal and adult stages of the two insects with their cross-specific responses. In addition, the compositions of volatile emissions of nymphal and adult stages of the two species were compared.

METHODS AND MATERIALS

Insects. Gregarious *L. migratoria migratorioides* and *S. gregaria* were reared under crowded conditions (100–200) in aluminum cages (50 × 50 × 50 cm) (Obeng-Ofori et al., 1993) in separate rooms (4.5 × 4.5 m each), which were well aerated with a duct system that maintained a negative pressure. Temperature was 30 ± 2°C, relative humidity was 60%, and the photoperiod was 12L : 12D. All stages were provided daily with fresh wheat seedlings and bran as food.

Olfactometric Assays. Aggregation responses of *L. m. migratorioides* and *S. gregaria* to airborne volatiles were conducted as described previously (Obeng-Ofori et al., 1993) in a single chamber olfactometer which eliminates visual and contact stimuli between the source and the test. Briefly, one side of the chamber was permeated with charcoal-filtered air from a compressed air cylinder enriched with volatiles from live locusts and the other only with the cleaned air. Air flow was 120 ml/min/side, and all experiments were conducted in a temperature controlled-room at 27 ± 2°C.

Sets of five each of mixed or separate sexes of 3- to 4 day-old fifth-instar nymphs and 20- to 40-day-old mature adults of *L. m. migratorioides* and *S. gregaria* were tested against their own and each other's volatiles at five different doses [3, 7, 10, 20, and 40 locust equivalents (LEQ)]. One locust equivalent represents the emission from one locust from a 2-liter flask for 10 min. The standard dose was set at 10 LEQ, and the dose responses were studied in the range of 0–10 and 10–50. Thus, in the lower range, doses 7 and 3 (decrease by 3 units) were tested, while in the upper range, doses 20 and 40 (increase by a factor of 2) were studied. Test insects were introduced into the olfactometer via the entry box attached to the front of the chamber. After 10 min, the number of insects on each side of the arena was recorded. Tests were replicated 10 times. The aggregation index (AI) for each test was calculated by using the equation $AI = 100\ (T - C)/N$, where T is the number of locusts found on the treated side, C is the number of locusts found on control side, and N is the total number of locusts tested (Obeng-Ofori et al., 1993).

Between experiments, the olfactometer was flushed several times with air to remove any volatile residues and cleaned with acetone. The source and control flasks were systematically switched between experiments to minimize bias.

Data Analysis. Differences between aggregation responses to fifth-instar nymphal and mature adult volatiles were compared using the t test ($P < 0.05$). Arcsine square-root-transformed means of aggregation responses to male and female volatiles of fifth-instar nymphs and mature adults were subjected to analysis of variance and compared using LSD test ($P < 0.05$) (SAS Institute, 1988).

Collection of Volatiles. Volatiles were collected separately from 25 males and 25 females of fifth-instar nymphs (3–4 days old) and mature adults (20–26 days old) of *L. m. migratorioides* and *S. gregaria.* A charcoal-filtered airstream (106

ml/min) from a compressed air cylinder was passed over the locusts contained in a 1-liter, three-necked, round-bottomed flask for 24 hr in a room maintained at 30 ± 2°C. The volatiles (insects plus feces) were collected on charcoal (60 mg) traps contained in Pyrex glass tubes (Torto et al., 1994). Fecal volatiles of nymphal stages of the two locust stages also were trapped separately for comparison since in *S. gregaria* they form part of the complex of the nymphal aggregation pheromone system (Obeng-Ofori et al., 1994b). The fecal volatiles were collected separately for 1 hr from 5 g of fresh feces obtained after feeding fifth-instar nymphs on fresh wheat shoots. Aerations of the feces were carried out in 12-cm-long × 2.5-cm-ID glass tubes. The volatiles were eluted from the charcoal traps with HPLC-grade dichloromethane (4 ml, Aldrich Ltd.), and stored in the freezer at −15°C until used.

Analysis of Volatiles. Volatiles were concentrated under a stream of nitrogen at 0°C to 100 μl. Eight microliters of the extracts were analyzed by gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975) on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with an FID detector and a Syntech EAD system by using conditions previously described (Torto et al., 1994). The GC oven was fitted with an HP capillary column (Carbowax 20 M, 50 m × 0.2 mm ID × 0.2 μm), and temperature programmed as follows: 60°C (10 min), 5°C/min to 180°C, held for 5 min, and raised at 10°C/min to 220°C. FID and EAD signals were monitored synchronously (Torto et al., 1996) by using a program on a GC/EAD interface card (Syntech) installed in a PC (Harvard Professional Computer, American Megatrends Inc.). The signals were recorded at a fixed speed of 9.2 cm/min and stored as pages of 128 sec duration. One complete run under these GC-EAD conditions generates 25 pages. In addition, the FID signal was plotted on an HP 3396 Series II integrator at 0.5 cm/min. GC-MS analysis was carried out on a Fisons VG-Platform II mass spectrometer (EI, 70 eV) coupled to a Fisons 8060 Series II gas chromatograph which employed the same GC oven conditions.

Volatiles analyzed by GC-EAD were replicated using four different antennae from mature adult males of *L. m. migratorioides* and *S. gregaria*. EAG-active compounds were identified by comparing their spectral data with those in the library (NIST Registry of Mass Spectral Data, 1995) of the mass spectrometer and confirmed with authentic samples in the case of (*E*)-2-pentenal, (*Z*)-2-pentenal, (*Z*)-2-hexenal, (*Z*)-2-pentenol, (*Z*)-2-hexenol, hexanoic acid, nonanoic acid, phenylacetonitrile, guaiacol, phenol, veratrole, 3,4-dimethoxystyrene (4-vinylveratrole), and indole (Aldrich, Ltd.).

RESULTS

Responses of L. m. migratorioides *to Their Own Volatiles.* Mature adults were indifferent (no significant difference from controls without nymphal odor)

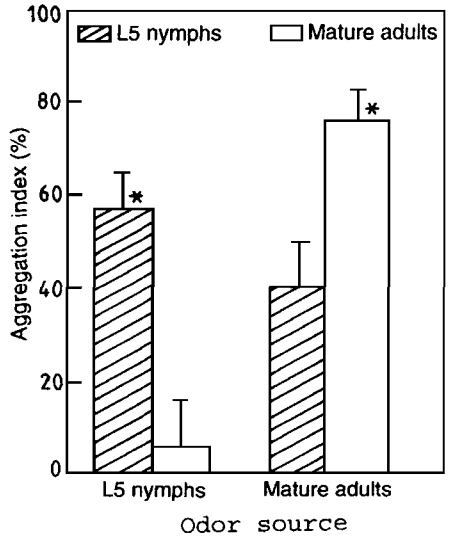

FIG. 1. Aggregation responses (mean ± SE) of *L. migratoria migratorioides* to airborne volatiles of live fifth-instar nymphs (L5) and mature adults at 10 LEQ in a choice olfactometer. Five locusts were tested per replicate and tests were replicated 10 times. *Significantly different at $P < 0.05$ (t test).

to nymphal volatiles, but responded strongly to their own (Figure 1). On the other hand, both fifth-instar nymphs and mature adults responded to the volatiles of the adults (Figure 1).

Responses of Different Sexes to Volatiles of L. m. migratorioides. Both male and female nymphs responded equally to their volatiles, showing that the aggregation stimulus is produced by both sexes (Figure 2). Likewise, both sexes of the adults produced an aggregation stimulus. However, females responded significantly more strongly to the male emission than males themselves; conversely, males were significantly more responsive to the volatiles of females than the latter to those of conspecifics (Figure 2).

Responses of L. m. migratorioides *to Their Own Volatiles and Volatiles of* S. gregaria. While fifth-instar nymphs of *L. m. migratorioides* responded in a dose-dependent fashion to the volatiles of conspecifics with a maximum at 20 LEQ, they responded to the volatiles of fifth-instar *S. gregaria* nymphs without discernible effect of the dose in the range tested (Figure 3A). On the other hand, the patterns of response of mature adults to intra- and interspecific volatiles were similar. However, maximum response to intraspecific volatiles occurred at 10 LEQ compared to 20 LEQ in inter-specific volatiles (Figure 3B).

Responses of S. gregaria *to Their Own Volatiles and Those of* L. m. migra-

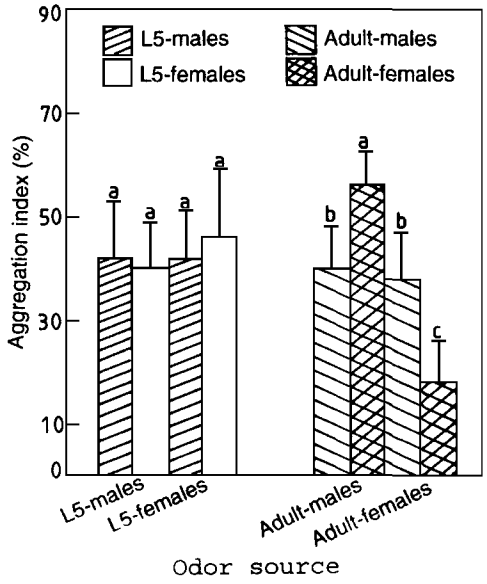

FIG. 2. Aggregation responses (mean ± SE) of *L. m. migratorioides* to airborne volatiles of live male and female fifth-instar nymphs (L5) and mature adults at 10LEQ in a choice olfactometer. Five locusts were tested per replicate and tests were replicated 10 times. Different letters indicate statistical differenoes between treatments, LSD test, $P < 0.05$.

torioides. Nymphal *S. gregaria* responses to their own volatiles and to those of *L. m. migratorioides* were similar (Figure 4A). However, the maximum in nymphal *S. gregaria* responses occurred at a lower dose (10 LEQ compared to 20 LEQ for nymphal *L. m. migratorioides* volatiles). Likewise, response patterns of adult *S. gregaria* to intra- and interspecific volatiles were similar, but interspecific responses were distinctly weaker at all doses above 7 LEQ (Figure 4B).

Analysis of Volatiles. Comparison of the volatile profiles of similar stages and sexes of *L. m. migratorioides* and *S. gregaria* showed significant quantitative and qualitative differences (Figures 5 and 6). Of *L. m. migratorioides* nymphal volatiles (Figure 5A), three compounds (hexanoic acid, phenylacetonitrile, and an unidentified compound, **1**) at natural concentrations consistently evoked strong EAG responses from male antennae of this locust species and those of *S. gregaria*. EAG responses were, however, stronger in *L. m. migratorioides* for hexanoic acid and phenylacetonitrile, while they were stronger in *S. gregaria* for compound **1**. Similar analysis of *S. gregaria* nymphal volatiles (Figure 5B), gave eight EAG-active compounds (hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid) by using male

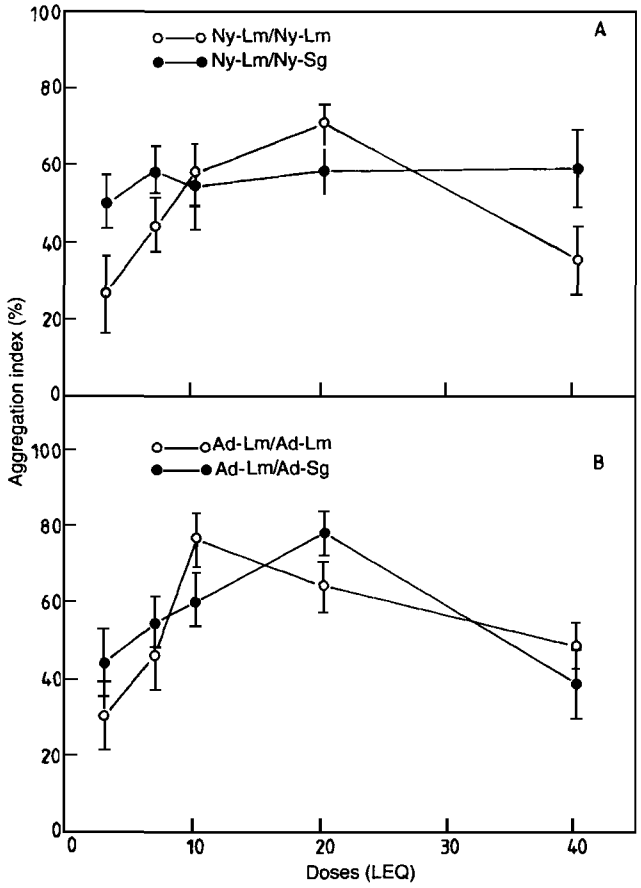

FIG. 3. Aggregation responses (mean ± SE) of (A) fifth-instar nymphs (Ny-Lm) of *L. migratoria migratorioides* to their own volatiles and those of conspecifics of *Schistocerca gregaria* (Ny-Sg) and (B) mature adults of *L. m. migratorioides* (Ad-Lm) to their own volatiles and volatiles of conspecifics of *S. gregaria* (Ad-Sg). Five locusts were tested per dose and tests were replicated 10 times.

antennae of this species (Torto et al., 1996). Only the acids evoked responses from male antennae of *L. m. migratorioides*. As previously reported for *S. gregaria*, there were no discernible differences in the volatile profiles of gregarious nymphal males and females of *L. m. migratorioides*.

Of the volatiles from adult *L. m. migratorioides* (Figure 6A), 14 compounds [nine identified as (*E*)-2-pentenal, (*Z*)-2-pentenal, (*Z*)-2-hexenal, (*Z*)-2-penten-1-ol, (*Z*)-2-hexen-1-ol, veratrole, hexanoic acid, guaiacol, and nonanoic acid;

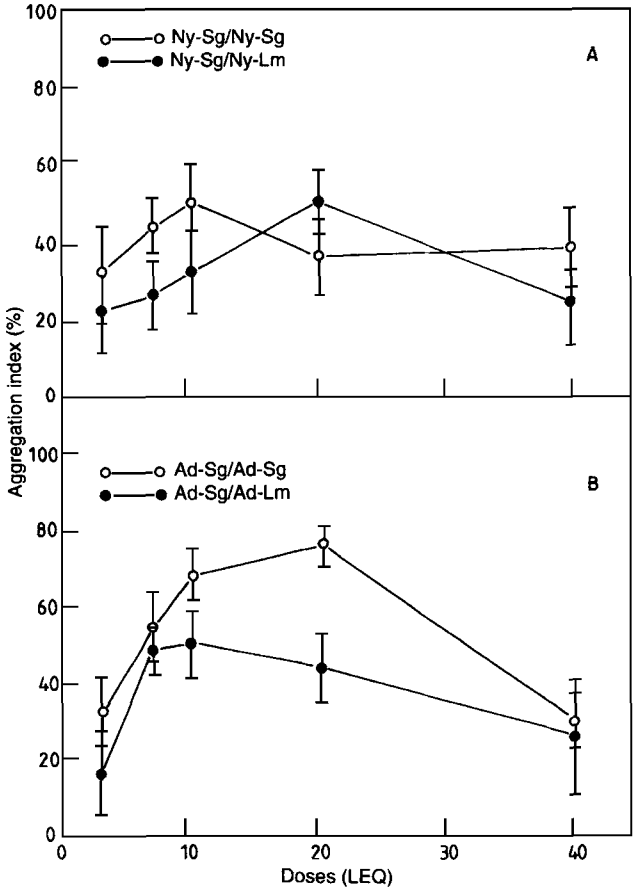

FIG. 4. Aggregation responses (mean ± SE) of (A) fifth-instar nymphs (Ny-Sg) of *Schistocerca gregaria* to their own volatiles and those of conspecifics of *L. migratoria migratorioides* (Ny-Lm) and (B) mature adults of *S. gregaria* (Ad-Sg) to their own volatiles and volatiles of conspecifics of *L. m. migratorioides* (Ad-Lm). Five locusts were tested per dose and tests were replicated 10 times.

and five aliphatic but not fully characterized components **1**, **2**, **3**, **4**, and **5**] evoked EAG responses from male antennae of this species, with veratrole evoking the strongest response. Of these, all except (*Z*)-2-hexen-1-ol and compound **3** at natural concentrations evoked EAG responses from *S. gregaria*. Overall, EAG responses evoked in *S. gregaria* to these components were stronger than in *L. m. migratorioides*. Of volatile components from adult *S. gregaria* (Figure 6B), five compounds (veratrole, guaiacol, phenylacetonitrile, phenol, and 4-vinylver-

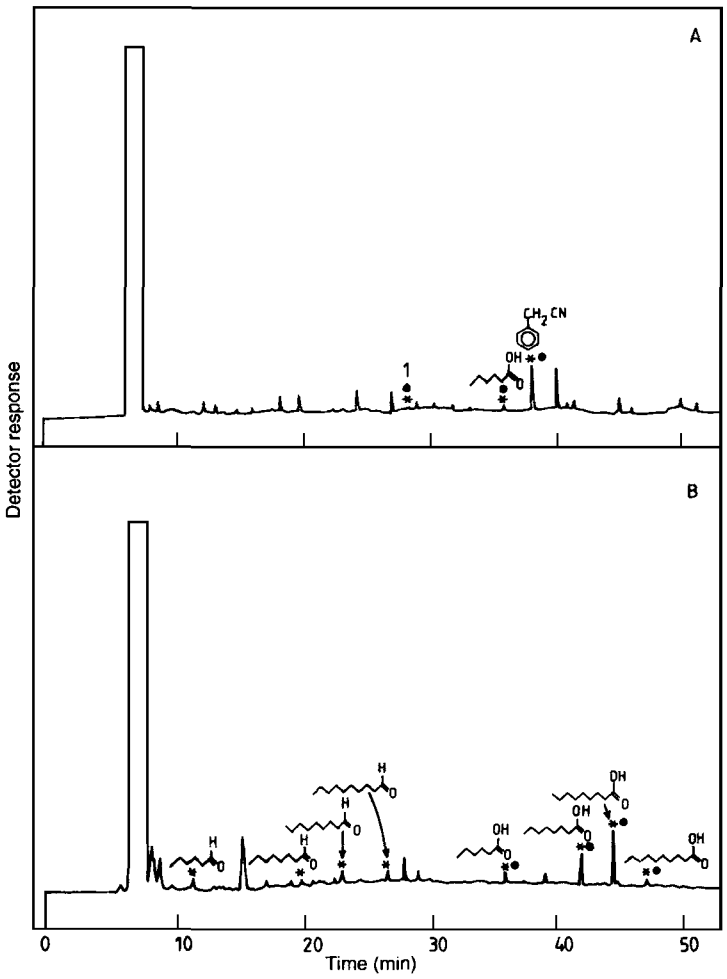

FIG. 5. Gas chromatograms of volatiles collected from fifth-instar nymphal (A) *Locusta migratoria migratorioides* and (B) *Schistocerca gregaria* injected onto a 50 m Carbowax 20 M capillary column. EAG-active peaks using antennae of (A) *L. m. migratorioides* (*), *S. gregaria* (•) and (B) *S. gregaria* (*), *L. m. migratorioides* (•).

atrole) at natural concentrations consistently evoked EAG responses from male antennae of this species (Torto et al., 1994). Of these, all except phenol at natural concentrations evoked EAG responses from *L. m. migratorioides* in addition to hexanoic acid and a compound identified as 7-octenoic acid based on its spectral characteristics (Figure 6B). Comparison of EAG responses showed that

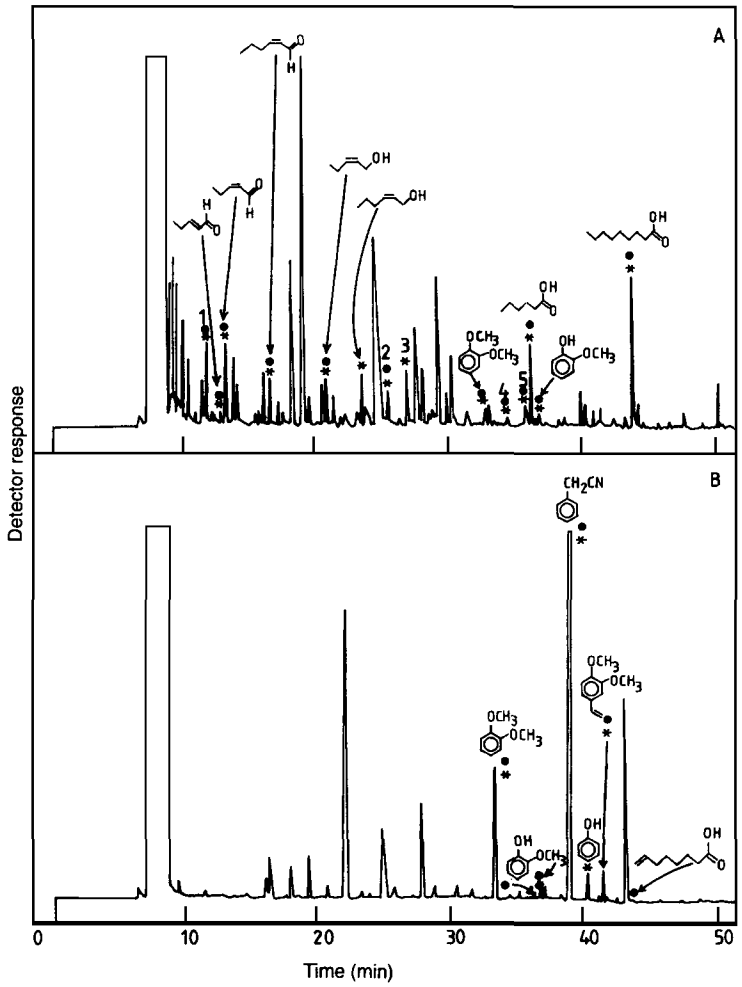

FIG. 6. Gas chromatograms of volatiles collected from adult (A) *Locusta migratoria migratorioides* and (B) *Schistocerca gregaria* injected onto a 50 m Carbowax 20 M capillary column. EAG-active peaks using antennae of (A) *L. m. migratorioides* (*), *S. gregaria* (•) and (B) *S. gregaria* (*), *L. m. migratorioides* (•).

overall they were stronger in *L. m. migratorioides* than in *S. gregaria* for the same set of compounds. Anisole and bezaldehyde in the volatile emissions of *S. gregaria* (Torto et al., 1994) evoked EAG responses in both locust species only at higher concentrations. Whereas in *S. gregaria*, adult pheromone production is male specific (Torto et al., 1994), both sexes of *L. m. migratorioides*

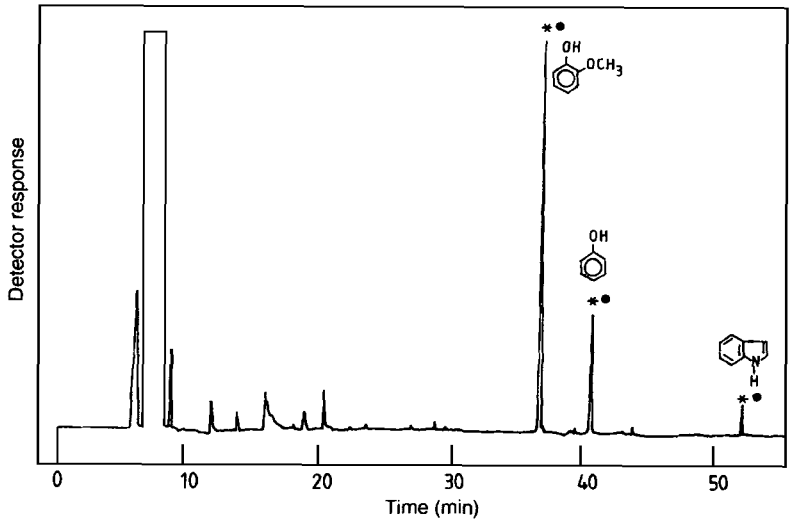

FIG. 7. Representative gas chromatogram of volatiles collected from feces of fifth-instar nymphs of *L. m. migratorioides* and *S. gregaria* and injected onto a 50 m Carbowax 20 M capillary column. EAG-active peaks using antennae of *L. m. migratorioides* (*) and *S. gregaria* (•).

adults produce aggregating signals, although that of males is compositionally richer.

Comparison of the volatile profiles of the feces of fifth-instar nymphs of *L. m. migratorioides* and *S. gregaria* showed no discernible differences. The major EAG-active compounds (Figure 7) were guaicaol and phenol, as well as indole, which was not detected in our earlier studies (Obeng-Ofori et al., 1994b) due to its lower volatility.

DISCUSSION

We previously reported that aggregation in gregarious-phase *S. gregaria*, involves stage- and sex-specific pheromones (Obeng-Ofori et al., 1993, 1994a; Torto et al., 1994, 1996). The results of the present study show a lesser pattern of pheromone differentiation in *L. m. migratorioides*. Thus, although nymphs do not respond significantly to the adult pheromone, adults responded positively to the nymphal pheromone (Figure 1), unlike *S. gregaria* adults, which did not show significant response to the corresponding nymphal pheromone (Obeng-Ofori et al., 1993). Similarly, whereas in *S. gregaria* adults production of the aggregation pheromone is male-specific (Obeng-Ofori et al., 1994a), in *L. m. migratorioides*,

both males and females induce significant level of aggregation from conspecifics (Figure 2).

Particularly noteworthy is the extent of cross-responses between the nymphal stages of the two species (Figures 1 and 2). Thus, the mixed hopper bands of the two species often observed in the field (Johnston and Buxton, 1949; El-Bashir and Abdel-Rahman, 1991) are not fortuitous events, but reflect strong interspecific aggregation responses to pheromone emissions of the two species. Among adults, although *L. m. migratorioides* was less discriminatory between its own and *S. gregaria* volatiles (Figure 1), *S. gregaria* adults were significantly more responsive to their own aggregation pheromone than to that of *L. m. migratorioides* (Figure 2). Interestingly, no mixed adult swarms of the two species have been reported in the field.

Comparative analyses of the adult volatiles of the two species (Figure 6A and B) allow some preliminary inferences to be made on the bases of interspecific aggregation responses observed between the corresponding stages of the two species. However, definitive conclusions must await detailed cross-aggregation bioassays of different blends of EAG-active components identified in the pheromone systems of the two species. Of *S. gregaria* pheromone components associated with second to fifth nymphal stages, C_6 and C_8 to C_{10} straight-chained aldehydes and acids, fecal phenols, guaiacol, and phenol (Torto et al., 1996), only the acids and phenols are present in *L. m. migratorioides*. These evoke varying levels of EAG responses in this insect with hexanoic acid being most active among the acids (Figure 5A). These compounds (acids and phenols) then constitute the common components of the nymphal stages of the two species and probably account for their cross-aggregation. Of special interest is the occurrence of phenylacetonitrile with prominent EAG activity in *L. m. migratorioides* nymphal volatiles. This compound is a major component of the adult *S. gregaria* pheromone but is absent in the corresponding nymphal volatiles (Torto et al., 1994, 1996). In *S. gregaria*, the nymphs' indifference to the pheromonal blends associated with older adults correlates directly with the presence of this component (Obeng-Ofori et al., 1994b; Torto et al., 1994; Ochieng, 1997). However, quantification of its emission has shown that an adult male *S. gregaria* produces about 300-fold more phenylacetonitrile than a male or female nymphal *L. m. migratorioides*. Thus, the relative amounts of this component in pheromone blends probably determine lack of aggregation of nymphal *S. gregaria* in one case but positive response in the other.

More distinct differences are found in the composition of adult volatile emissions of the two species (Figure 6A and B). In *S. gregaria* adults, volatile emissions are comprised wholly of benzene derivatives (Mahamat et al., 1993), of which phenylacetonitrile, guaiacol, phenol, and benzaldehyde make up the aggregation blend (Torto et al., 1994). Others, such as veratrole and anisole, were electrophysiologically active but behaviorally inactive in aggregation assays. The

present study has shown that *L. m. migratorioides* adult volatiles are dominated by aliphatic compounds reminiscent of Graminae volatile components (Njagi and Torto, 1996). Veratrole and guaiacol are the only EAG-active benzene derivatives present in significant amounts. This would account for the significantly lower aggregation responses of *S. gregaria* to *L. m. migratorioides* adult volatiles.

In conclusion, our results with laboratory-reared insects demonstrate a lesser pattern of stage and sex differentiation in the aggregation pheromone system of *L. m. migratorioides* compared to that of *S. gregaria*. The two species, and particularly their nymphal stages, respond to each other's pheromone emissions and account for the mixed hopper bands often observed in the field. The two also share some common components in their emissions. Further studies with laboratory and wild insects are needed to identify precisely the compounds involved and their cross-aggregative efficiency. An intriguing question is whether this interaction is incidental to pheromone composition or whether it represents a form of protocooperation (Odum, 1971) that is beneficial to the two whenever opportunity arises. Since gregarization is a density-driven process, and both solitary as well as gregarious insects respond to gregarious-phase aggregation pheromones (Njagi et al., 1996), it is conceivable that such interactions may facilitate phase transformation at lower densities of each species and confer attendant benefits to both. Further studies are needed to shed light on this question.

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HOST-PLANT CHEMICALS AND DISTRIBUTION OF *Neuquenaphis* ON *Nothofagus*

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Abstract—Extractable metabolites from leaves (EM) and volatiles released from six *Nothofagus* species were analyzed by TLC and GC-MS, respectively. Aphids of the genus *Neuquenaphis*, closely associated to *Nothofagus*, were sampled on each *Nathofagus* species. Cluster analyses of *Nothofagus* species were performed based on the presence or absence of EM and volatiles. Dissimilarity distances, from the cluster analyses of EM and volatiles, were used to evaluate their association with the aphid distribution. A major component identified from EM and volatiles of three species of *Nothofagus*, α-agarofuran, was attractive to alates of the oligophagous *Neuquenaphis sensoriata*, which use them as hosts. These results suggest that chemicals play a significant role in the host-plant associations between *Neuquenaphis* and *Nothofagus*.

Key Words—*Nothofagus*, *Neuquenaphis*, volatiles, olfactometry, α-agarofuran.

INTRODUCTION

The genus *Nothofagus* (Fagaceae), the southern beech, comprises about 35 living species distributed in South America, Australia, New Zealand, New Caledonia, and Papua New Guinea (Poole, 1987). This disjunct distribution constitutes a canonical example of biogeographical patterns explained by the continental drift hypothesis (Poole, 1987). In Chile, 10 species belonging to this genus have been

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described (Ramírez, 1987). They account for nearly half of the area covered by native forests (Carrillo and Cerda, 1987).

The biogeographic relevance of this genus (Schlinger, 1974; McQuillan, 1993; Starý, 1994; Burckhardt, 1997) as well as its economic significance for forestry (Carrillo and Cerda, 1987) has promoted the study of its entomofauna. Among herbivore guilds, sap-suckers show one of the highest species richness (McQuillan, 1993). In particular for South America, aphids, represented by the genus *Neuquenaphis* (Hemiptera: Aphididae), are a major component of this diversity (McQuillan, 1993; Quednau and Remaudière, 1994).

A recent review of the aphid fauna from Chile reported that *Nothofagus glauca* (Phil.) Krasser, *Nothofagus obliqua* (Mirb.) Oerst. var. *obliqua*, and *Nothofagus dombeyi* (Mirb.) Oerst. share five of the 12 extant *Neuquenaphis* species, while the seven remaining aphid species are mainly monophagous on different *Nothofagus* species (Fuentes-Contreras et al., 1997).

During host selection by aphids, visual and olfactory stimuli play an important role in host recognition, while epidermal and internal constituents are important during host acceptance (Niemeyer, 1990). Thus, laboratory and field studies have demonstrated the response of aphids to plant volatiles (Pettersson, 1970; Campbell et al., 1993; Nottingham and Hardie, 1993; Pettersson et al., 1995; Quiroz and Niemeyer, 1997), chemical and physical characteristics of the plant surface (Klingauf, 1987), and extractable chemical constituents (Niemeyer, 1991). Furthermore, volatiles (Jackson *et al.*, 1996; Jackson and Dixon, 1996) and internal secondary metabolites (Zucker, 1982) explain the distribution of aphids on different microhabitats within a tree.

The reported patterns of *Neuquenaphis–Nothofagus* associations led us to initiate research that explores the role of chemical factors, particularly the leaf volatiles of the six main *Nothofagus* species in central Chile, in determining the distribution of *Neuquenaphis*.

METHODS AND MATERIALS

Study Sites. During December 1996, aphid and chemical samplings were performed in two protected areas near Talca, Chile: (a) Altos de Lircay National Reserve (35°37′S; 71°03′W), where five species of *Nothofagus* [*No. glauca, No. obliqua, No. dombeyi, No. pumilio* (P. et E.) Krasser and *No. antarctica* (G. Forster) Oerst.] were present and studied, and (b) Los Ruiles National Reserve (35°49′S: 72°31′W), where *No. alessandrii* Esp. was studied.

Aphid Sampling. Five sampling sites were designated, and the aphid distribution was evaluated in all *Nothofagus* species present at each site. Three branches of each tree were randomly chosen, and the aphids present in the apical 15 cm of each branch were counted. Aphids from every tree sampled were

collected and kept in 70% ethanol for later taxonomic identification, according to the key of Quednau and Remaudière (1994).

Entrainment of Nothofagus Volatiles. The apical 30 cm of a branch 1 m above the ground of each *Nothofagus* species was enclosed in a plastic bag. Air entering the bag was purified by passage through a filter with activated charcoal and exited the bag through a filter containing Porapak Q. The air was drawn with an oil-free pump at 1 liter/min for 1 hr. A blank trapping was carried out in each site, 1 m from the sampled branch. The trapped volatiles were desorbed from the Porapak Q with distilled diethyl ether (2.5 ml) and concentrated (200 μl) under a stream of nitrogen to give an extract containing the leaf "volatiles."

Analysis of Volatiles. Aliquots (1 μl) of the concentrated volatile extracts were analyzed by GC-MS. A capillary Ultra-2 HP GLC column (25 m × 0.2 mm ID) was directly coupled to a mass detector with an integrated data system (GC model HP-5890, MD model HP-5972). Ionization was by electron impact at 70 eV and 280°C. The GC oven was programmed to remain at 40°C for 4 min and then increased at 5°C/min to 300°C. A library was constructed of the retention time and mass spectra of all compounds detected in the volatile extracts of each *Nothofagus* species studied. The presence or absence of a given compound in the profile of each *Nothofagus* species was determined by comparing retention times and mass spectra with this library. The GC peaks were considered to be coincident and the compounds identical if their retention times did not differ by more than ±0.03 min and the similarity index of their mass spectra (Pensyna, 1976) was higher than 95%. The components of the volatile extract were identified by comparing their GLC Kováts indexes and mass spectra with those of commercial standards. Where standards were not available, spectra were either compared with a library database by a reverse search technique, which verifies that the main peaks in a reference spectrum are present in the unknown spectrum (Pensyna, 1976), or by analysis of the mass fragmentation patterns.

Extraction of Nonvolatiles. Young leaves of each *Nothofagus* species were collected, air-dried, ground, and extracted (70 g) with CH_2Cl_2 (0.5 liter) for 20 min. The mixture was filtered, and the residue reextracted with MeOH (0.5 liter) for 20 min. Both extracts were concentrated to dryness in a rotary evaporator. Each extract was dissolved in acetone, and aliquots of these solutions (corresponding to 100 mg of dry plant material) were analyzed by TLC (Silica gel F-254) by development with CH_2Cl_2 (for the CH_2Cl_2 extract) and MeOH/CH_2Cl_2 (1 : 9) (for the MeOH extract). The TLC plates were visualized with Liebermann reagent, vanillin/H_2SO_4, or phosphomolybdic acid. A table of the presence or absence of extractable metabolites was produced in terms of R_f values.

*α-Agarofuran (Compound **30**).* Dry leaves (100 g) of *No. glauca* were steam distilled. The aqueous distillate was extracted with CH_2Cl_2 (×3) and the organic phase concentrated to a pale yellow oil (0.5 g). The oil was subjected to chromatography on silica gel, with a stepwise hexane–CH_2Cl_2 gradient. Pure

α-agarofuran (50 mg) was eluted with hexane–CH_2Cl_2 (55 : 45) as an oil, $[\alpha]_D$ = +36.4° ($CHCl_3$, c = 2.5), lit. +41.5° (Barrett and Buchi, 1967). ^1H NMR (300 MHz, d_6-benzene) δ: 5.55 (1H, br s), 2.06 (1H, ddd), 1.97 (1H, dd), 1.90 (2H, m), 1.71 (3H, s), 1.70 (1H, dd), 1.66 (1H, dd), 1.54 (2H, m), 1.49 (1H, d), 1.29 (3H, s), 1.14 (3H, s), 1.09 (1H, ddd), 0.99 (1H, dd), 0.86 (3H, s). ^{13}C NMR δ: 133.3 (C-4), 126.7 (C-3), 84.7 (C-5), 80.6 (C-11), 44.6 (C-7), 37.3 (C-10), 34.8 (C-9), 33.2 (C-1), 32.7 (C-6), 30.5 (C-12), 24.8 (C-8), 23.0 (C-2), 23.0 (C-13), 22.0 (C-15), 19.3 (C-14). El-MS m/z (rel. int. %): 220 (M^+, $C_{15}H_{24}O$, found, 220.1833, requires, 220.1827, 87), 205 (28), 202 (21), 187 (11), 177 (9), 162 (12), 147 (34), 131 (11), 124 (14), 123 (36), 122 (14), 121 (14), 119 (12), 109 (32), 107 (12), 105 (22), 95 (15), 91 (25), 82 (100), 55 (22), 43 (30), 41 (46).

Olfactometry. Behavioral studies were performed in an olfactometer as described by Pettersson (1970). The olfactometer consisted of a quadratic arena permeated by air (250 ml/min) coming from the stretched-out corners (arms) and drawn on through a hole above its center. Two glass tubes containing the same single chemical stimulus were connected to the end of two consecutive arms; tubes containing control stimulus (hexane) were connected to the other two arms. The observation arena was divided into four arm zones and one indifferent zone in the center. A field-collected test aphic (*Neuquenaphis sensoriata*, alate and apterous, and *Ne. bulbicauda*) was placed in the center of the arena and the time the aphid spent in each arm was recorded during 15 min with the olfactometer being rotated by 90° every minute. Each experiment was replicated 10 times and results analyzed by nonparametric statistics (Wilcoxon one-tailed rank-sum test for two groups); the mean time spent in the treatment arms was compared with the mean time spent in the control arms.

Dissimilarity and Correlation Analyses. For each *Nothofagus* species, the presence or absence of EM, volatiles, and *Neuquenaphis* species was tabulated, and cluster analysis performed to compare the chemical profiles with aphid distribution. Distance measures between species were calculated according to the dissimilarity algorithm 1 – (Pearson's r), which assigns a value of 0 to maximum similarity and a value of 1 to minimum similarity. Clusters of species were estimated by using unweighed pair-group of arithmetic average (UPGMA) as a linkage rule (Manly, 1994). Subsequently, the values of distance obtained from the cluster analysis of volatiles and internal secondary metabolites were separately used to estimate simple Kendall's correlations with aphid distribution (Siegel and Castellan, 1988).

RESULTS AND DISCUSSION

The distribution of aphids observed on the six *Nothofagus* species studied is shown in Table 1. This distribution generally agrees with data reported in

TABLE 1. AVERAGE NUMBER OF APHIDS PER BRANCH OBSERVED ON SIX *Nothofagus* SPECIES IN CENTRAL CHILE

	Nothofagus (mean ± SD)						Previous
Neuquenaphis	*glauca*	*obliqua*	*dombeyi*	*pumilio*	*antarctica*	*alessandrii*	reports[a]
sensoriata	7.8 ± 16.7	7.8 ± 9.7			4.5 ± 7.7		*No. glauca* *No. obliqua*
bulbicauda			2.0 ± 6.9				*No. dombeyi* *No. obliqua*[b]
similis				10.4 ± 12.9			*No. pumilio* *No. obliqua*[c]
staryi						0.5 ± 0.5	*No. alessandrii*

[a]Information from Fuentes-Contreras et al. (1997).
[b]British Museum of Natural History, possible wrong identification of the host plant.
[c]Hille Ris Lambers (1968), possible wrong identification of the host plant.

the literature (Quednau and Remaudière, 1994; Fuentes-Contreras et al., 1997), although this is the first report of *Ne. sensoriata* occurring on *No. antarctica*. The eventual exceptions to the general agreement with previously published data are *Ne. bulbicauda* Hille Ris Lambers and *Neuquenaphis similis* Hille Ris Lambers. In the first instance, a single record at the British Museum of Natural History (BMNH) collection indicates that *Ne. bulbicauda* was collected on *No. obliqua*. However, all other records of this species in the BMNH collection (72 mounted specimens) and information from the literature (Fuentes-Contreras et al., 1997) report this aphid on *No. dombeyi*. In the second instance, *No. obliqua* was originally mentioned as a host plant for *Ne. similis* (Hille Ris Lambers, 1968). Since all the subsequent collections of *Ne. similis*, including the present one, have been obtained form *No. pumilio* (Blackman and Eastop, 1994), it is likely that the six specimens of the type series (one holotype and five paratypes) of *Ne. similis* were labeled incorrectly by their original collectors. Neither *Ne. similis* nor *Ne. bulbicauda* were observed on *No. obliqua* in spite of the coexistence of these three species in a same sampling site.

From the GC and TLC profiles of the volatile and extractable metabolites, respectively, cluster analysis enabled dendrograms of the six *Nothofagus* species to be constructed. A nonsignificant Kendall's correlation ($\tau = 0.31$, $N = 14$, $P = 0.10$) was found between aphid distribution and the TLC profile of extractable leaf metabolites (principally nonvolatiles). Similarly, a nonsignificant Kendall's correlation ($\tau = -0.06$, $N = 15$, $P = 0.74$) was found between aphid distribution and the GC profile of the entrained leaf volatiles. Although the probability values are not significant at the 5% level in either case, comparison of their magnitudes suggests a weak but closer to significant correlation with the extractable metabolites profile and aphid distribution. However, the correlations

of extractable metabolites and volatiles may not be absolutely independent since components in one group may also occur in the other group.

The identity and relative abundance of the constituents of the entrained volatiles collected from the foliage of each *Nothofagus* species are shown in Table 2. The compounds listed can be categorized into three groups. The first group consists of commonly found leaf volatiles (Knudsen et al., 1993), *viz*, monoterpenes (compounds **1**, **2**, **3**, **9**, **10**, and **12** in Table 2), methylheptenone (**6**), hexene esters (**7** and **8**), aldehydes (**4**, **13**, and **18**), phenolics (**5**, **11**, and **18**), and ethyl octanoate (**16**). The second group, corresponding to ca. 46% of all compounds listed in Table 2 are provisionally identified as sesquiterpenes (**19–27**, **29–34**, and **36**). Some of these compounds are common plant constituents, e.g., α-cubebene, copaene, and caryophyllene, while others have restricted occurrence. The third group of volatiles are those compounds (**14**, **15**, **28**, and **35**) that remain to be identified. Both the relative importance of these three groups and the abundance of individual compounds were highly variable between *Nothofagus* species.

The major sesquiterpene occurring in the volatile profile of *No. obliqua*, *No. glauca*, and *No. antarctica* was identified as α-agarofuran (5,11-oxy-5β,10α-eudesm-3-ene, **30**) (Figure 1), a sesquiterpene previously isolated from agarwood (*Aquillaria agallocha*) (Barrett and Buchi, 1967). α-Agarofuran was readily isolated from a steam distillate of dried leaves of *No. glauca* and its identity established from careful analysis of its proton and carbon NMR spectra. A 2D-COSY experiment quickly established carbon–proton connectivities, and the eudesmane skeleton and position of the oxy ring was established from a Heteronuclear Shift Correlation Multiple Bond Connectivities experiment. A Nuclear Overhauser Enhancement of the C-15 angular methyl on irradiation of the C-6 proton ($\triangleq$ 1.46) showed that the ring junction was *trans*, consistent with **30**.

The occurrence of α-agarofuran in *No. obliqua*, *No. glauca* and *No. antarctica*, and its absence in *No. dombeyi*, correlates with the former trees being host-plants and the latter tree a nonhost of the oligophagous *Ne. sensoriata*. Therefore, α-agarofuran is a good candidate for studying the role of host-plant chemicals in the orientation behavior of these aphids. Olfactometric bioassays showed that alate morphs of *Ne. sensoriata* were significantly attracted by α-agarofuran compared to the solvent control (Table 3), but the apterous morph did not show a significant response. Such differential response between alate and apterous morphs has been reported for other aphid species (Quiroz and Niemeyer, 1997). Apterous individuals of the monophagous *Ne. bulbicauda*, which associate with *No. dombeyi*, a species lacking α-agarofuran, was not significantly affected by α-agarofuran in olfactometric tests (Table 3). The alate morph of *Ne. bulbicauda* was not observed in the field and has not been reported in the literature (Hille Ris Lambers, 1968; Quednau and Remaudière, 1994; Blackman and Eastop, 1994). It could not be tested.

TABLE 2. COMPOSITION OF VOLATILES FROM SIX *Nothofagus* SPECIES[a]

Peak and Cmpd No.	Compound	Identification method[b]	GLC Kovats index Ultra-2	Relative areas (%)					
				N. o.	N. g.	N. d.	N. an.	N. al.	N. p.
1	3-Carene	B	920	nd	nd	3.6	nd	nd	nd
2	α-Pinene	A	933	nd	nd	23.2	nd	nd	1.9
3	Camphene	A	949	nd	nd	19	nd	nd	1.1
4	Benzaldehyde	A	960	0.8	0.2	2.6	4.4	8.5	1.1
5	Phenol	A	977	0.3	nd	7.5	8.2	9.9	7.5
6	6-Methyl-5-hepten-2-one	A	984	nd	nd	5.3	4	6.5	nd
7	(*Z*)-3-hexenyl acetate	A	1004	40.1	2.4	nd	9.9	22.4	14.4
8	Hexyl acetate	A	1009	0.7	0.4	nd	4.1	nd	nd
9	Limonene	A	1030	nd	nd	nd	1.4	3.6	12.5
10	(*E*)-3,7-Dimethyl-1,3,6-octatriene	B	1046	0.9	0.1	nd	6.6	nd	nd
11	Acetophenone	A	1068	nd	nd	nd	nd	2.4	nd
12	Linalool	A	1089	3.1	0.5	2.3	nd	nd	nd
13	Nonanal	A	1102	0.7	0.4	5.7	6	8.7	1.5
14	Unknown 1 M_r 150 (*m/z*:41, 53, 69,[c] 81, 107, 121, 135)		1114	0.4	nd	nd	2.8	nd	nd
15	Unknown 2 M_r 115 (*m/z*:29,[c] 44, 56, 72, 86, 100)		1129	nd	nd	nd	nd	nd	5.1
16	Ethyl actanoate	B	1193	1.3	nd	nd	2.5	3.8	2
17	Decanal	A	1203	0.4	0.3	3.2	2.8	4.1	0.7

TABLE 2. CONTINUED

Peak and Cmpd No.	Compound	Identification method[b]	GLC Kovats index Ultra-2	Relative areas (%)					
				N. o.	N. g.	N. d.	N. an.	N. al.	N. p.
18	4-Ethylacetophenone	B	1267	nd	0.4	nd	1.4	nd	nd
19	α-Cubebene	A	1356	nd	0.6	nd	nd	nd	nd
20	α-Amorphene	B	1378	nd	0.8	nd	nd	nd	nd
21	Copaene	A	1386	0.2	10.6	nd	4	nd	nd
22	Sesquiterpene M_r 204 (*m/z*: 41, 55, 57, 77, 91, 105,[c] 119, 133, 147, 161, 175, 189)	B	1421	2.2	nd	nd	nd	nd	nd
23	Caryophyllene	A	1437	1.3	20.4	nd	nd	nd	nd
24	Sesquiterpene M_r 204 (*m/z*: 41,[c] 55, 69, 79, 91, 105, 119, 133, 147, 161, 189)	B	1475	10.7	1.8	nd	nd	nd	2.7
25	Sesquiterpene M_r 204 (*m/z*: 41, 55, 67, 81,[c] 91, 107, 121, 133, 147, 161, 189)	C	1484	1.1	nd	nd	nd	nd	nd
26	Sesquiterpene M_r 204 (*m/z*: 41, 55, 67, 80, 91, 105,[c] 121, 133, 147, 161, 189)	C	1497	0.6	nd	nd	nd	nd	nd
27	Saturated naphthalene M_r 204 (*m/z*: 41, 55, 67, 79, 91, 105, 119, 133, 147, 161,[c] 189)	B	1507	6.7	14.7	nd	4	nd	0.5
28	Unknown 3 M_r (*m/z*: 43,[c] 55, 69, 81, 95, 109, 123, 137, 149, 161, 189)		1519	nd	0.6	nd	nd	nd	nd

29	Sesquiterpene M_r 204 (*m/z*: 41, 55, 67, 79, 91, 105, 119, 133, 147, 161,[c] 189)	C	1536	0.3	1.6	nd	nd	nd	nd
30	α-Agarofuran	A	1567	19.9	38.7	nd	26	nd	nd
31	Sesquiterpene M_r 204 (*m/z*: 43,[c] 55, 69, 81, 91, 107, 121, 133, 147, 161, 189)	C	1603	nd	nd	nd	nd	nd	7.6
32	Sesquiterpene M_r 204 (*m/z*: 43,[c] 55, 67, 80, 91, 105,[c] 121, 133, 147, 161, 189)	C	1612	nd	nd	3.5	nd	nd	13.7
33	Sesquiterpene M_r 204 (*m/z*: 43,[c] 55, 69, 81, 91, 107, 122, 133, 147, 161, 189)	C	1623	1.1	nd	nd	nd	nd	nd
34	Saturated naphthalene M_r 204 (*m/z*: 59, 77, 105, 119, 135, 149, 161,[c] 189)	C	1672	1.5	0.8	nd	nd	nd	nd
35	Unknown 4 M_r 218 (*m/z*: 41, 55, 65, 77, 91, 105, 115, 128, 135,[c] 149, 162, 175, 203)		1785	3.7	1.5	24.2	11.9	30	27.7
36	Saturated naphthalene M_r 204 (*m/z*: 43, 55, 67, 79, 91, 105, 119, 133, 147, 161,[c] 189)	C	1795	2	1.3	nd	nd	nd	nd
Relative total area[d]				3.56	6.11	1.46	1.40	1.39	2.34

[a]As percent of total volatile compounds. N. o. = Nothofagus obliqua var. obliqua, N. g. = No. glauca, N. d. = No. domeyi, N. an. = No. antarctica, N. al. = No. alessandrii, N. p. = No. pumilio, nd = not detected.

[b]Identification codes: A = comparison with authentic compounds; B = GC-MS comparison between the recorded and library mass spectra with a similarity index higher than 95%; C = tentative identification based on fragmentation patterns in the mass spectra.

[c]Main peak.

[d]Calculation based on total area/area of internal standard (docosane).

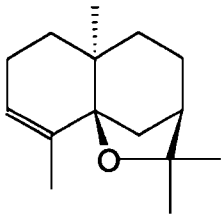

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FIG. 1. 5,11-Oxy-5β,10α-eudesm-3-ene, or α-agarofuran, the major sesquiterpene occurring in the volatile profile of *Nothofagus obliqua*, *Nothofagus glauca*, and *Nothofagus antarctica*.

TABLE 3. RESPONSE OF *Neuquenaphis* INTRODUCED SINGLY INTO AN OLFACTOMETER AND EXPOSED TO 10 μg OF α-AGAROFURAN AND HEXANE

Test aphid	Time spent (min)[a]		P^b
	Hexane	10 μg of α-agarofuran	
N. sensoriata alatae	4.9 ± 1.7	8.0 ± 1.1	0.01
N. sensoriata apterae	5.5 ± 1.5	6.4 ± 1.8	0.20
N. bulbicauda apterae	7.9 ± 3.3	4.6 ± 2.8	0.09

[a]Mean ± standard error.
[b]Wilcoxon one-tailed rank-sum test for two groups.

In summary, the results show that patterns of host-plant volatiles emitted from leaves, and α-agarofuran in particular cases, may play a significant role in host orientation of aphids that feed on *Nothofagus*. Careful identification and testing of the remaining *Nothofagus* volatile compounds with *Neuquenaphis* will be required before a complete picture can emerge. The nonvolatile or extractable metabolites undoubtedly play a role in host acceptance and the correlations described in this work, although weak, will need to be confirmed by detailed chemical and behavioral assays.

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ENANTIOMERIC ANOSMIA IN SCARAB BEETLES

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Abstract—The sex pheromone for *Anomala octiescostata* has been previously identified as a 4 : 1 mixture of (*R*)-buibuilactone and (*R*)-japonilure. Field experiments showed that although not active per se, the secondary component increased the activity of major component, buibuilactone. The enantiomers of these semiochemicals, i.e., (*S*)-buibuilactone and (*S*)-japonilure are neither attractants nor behavioral antagonists to the pheromone system of *A. octiescostata*. Chiral GC-EAD experiments demonstrated that, in marked contrast to the Japanese beetle and Osaka beetle, which can detect a stereoisomer not produced by their conspecific females, the male antennae of *A. octiescostata* lack olfactory receptor neurons (ORNs) tuned to (*S*)-buibuilactone. The same enantiomeric anosmia (inability to smell a compound) was also observed in *Anomala cuprea* male antennae. Both in a Y-olfactometer and field experiments, it was clarified that (*S*)-buibuilactone was not a behavioral antagonist for *A. cuprea*. Contrary to male antennae of both *A. octiescostata* and *A. cuprea*, which lack ORNs tuned to the nonnatural (*S*)-buibuilactone, fragrance evaluation by skilled perfumers revealed that the human nose can discriminate (both in intensity and quality) enantiomerically pure samples of (*R*)- and (*S*)-buibuilactone and japonilure.

Key Words—Chiral GC-EAD, enantiomeric discrimination, *Anomala octiescostata*, *Anomala cuprea*, buibuilactone, japonilure.

INTRODUCTION

The importance of chirality, or left- and right-handedness, in chemosensory processes was demonstrated over a century ago by a study of gustatory discrimination between enantiomers of asparagine in humans, the (+) isomer being sweet, while the (−) isomer has an insipid taste (Piutti, 1886). Many other cases of similar enantiomeric differences, both in olfactory and gustatory perception, have been reported. After the tragedy of thalidomide (Fabro, 1981), chirality became

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a more important issue in medicinal chemistry. The suggestion that oral administration of a single enantiomer of thalidomide could have prevented the teratological effects associated with the *S* enantiomer (Faigle et al., 1962; Ockenfels and Kohler, 1970; Kohler et al., 1971), although probably incorrect (Fabro et al., 1967; Wnendt and Zwingenberger, 1997), has led to the "racemic switch" (Stinson, 1993), i.e., the redevelopment of single enantiomers from older chiral drugs originally marketed as racemates.

The fact that animals, including insects, can distinguish between different volatile chemical compounds (for example *cis* and *trans* stereoisomers) was never doubted, but until the issue was settled conclusively in 1971, there was some controversy as to whether enantiomers were different enough from one another for humans to distinguish them by odor (Silverstein, 1979). In that year, three independent groups prepared highly purified samples of the carvone enantiomers and showed by panel tests that the odors differed in intensity and character. (−)-Carvone has a spearmint odor and is detectable at lower threshold than (+)-carvone, which has a caraway odor (Friedman and Miller, 1971; Russel and Hills, 1971; Leitereg et al., 1971).

In insects, the importance of stereochemical discrimination with respect to *E* and *Z* isomerism was recognized with the characterization of bombykol (Butenandt and Hecker, 1961). The existence of chiral pheromones was first recognized in the pioneering works of Silverstein (e.g., Silverstein et al., 1966) and in a few studies of enantiomeric effects on insect behavior (reviewed in Seybold, 1993). Later, Tumlinson et al. (1977) demonstrated that the presence of a nonnatural enantiomer dramatically reduced the response of *Popillia japonica* (Japanese beetle) males to the female-released sex pheromone, (*R*)-japonilure (Figure 1C). The recent discovery of the sex pheromone of the Osaka beetle, *Anomala osakana*, disclosed a very interesting mechanism of mutual behavioral agonist–antagonist activities of enantiomeric pheromones (Leal, 1996). The Osaka beetle, which shares with the Japanese beetle the same habitats in parts of the mainland of the Japanese archipelago, produces and responds to (*S*)-japonilure (Figure 1D). The Japanese beetle sex pheromone, (*R*)-japonilure, shuts down male response in the Osaka beetle. Chiral gas chromatography with an electroantennographic detector (Leal et al., 1995) demonstrated that both the Japanese and Osaka beetles had olfactory receptor neurons tuned to each enantiomeric form of japonilure (Leal, 1996). More recently, it was demonstrated by the single sensillum recording technique that the olfactory receptor neurons in the antennae of the Japanese beetle and the Osaka beetle are extremely specific to each enantiomeric form of the semiochemical. Receptors tuned to one enantiomer did not respond to the antipode even when challenged with a 1000-fold higher concentration (Wojtasek et al., 1998). These findings prompted me to investigate whether the behavioral antagonism and the occurrence of olfactory receptor neurons (ORNs) specifically tuned to the nonnatural enantiomeric

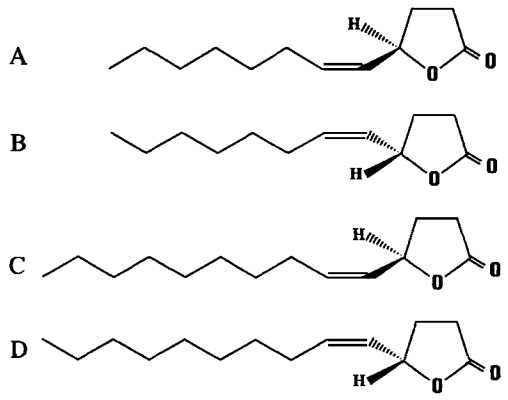

FIG. 1. Structures of (*R*)-buibuilactone (A), (*S*)-buibuilactone (B), (*R*)-japonilure (C), and (*S*)-japonilure (D).

form of the pheromone were common features in the chemical communication of scarab beetles. Here, I report that two species of scarab beetles, *A. octiescostata* and *A. cuprea*, for which (*R*)-buibuilactone (Figure 1A) is the major constituent of their pheromone systems (Leal, 1991; Leal et al., 1993, 1994), do not have ORNs tuned to the *S* enantiomer (Figure 1B), which is neither a behavioral antagonist nor beneficial for pheromonal activity. Interestingly, humans can discriminate the enantiomers of buibuilactone, both in odor quality and intensity.

METHODS AND MATERIALS

Chiral Gas Chromatography with Electroantennographic Detection. Resolution of pheromone enantiomers was achieved on a capillary column having a trifluoroacetylated γ-cyclodextrin phase, Chiraldex GTA (20 m × 0.25 mm; 0.125 μm; Astec, Whippany, New Jersey) operated at 130°C and with helium head pressure at 2.4 kg/cm^2 (10 ml/min). The plumbing of the GC-EAD has been previously described (Leal et al., 1996), and the electronics for the amplifier and filter were as previously reported (Leal et al., 1995; Leal, 1996).

Pheromones. (*R*)- and (*S*)-Buibuilactone and japonilure (Figure 1) were synthesized from D- and L-glutamate following the procedure of Tumlinson et al. (1977). The enantiomeric purity was improved according to a previously reported method (Ono et al., 1994). In brief, buibuilactone (11 g) was added to dry methanol containing Na (1.1 g) and stirred for 1 hr. Water (30 ml) was added and the mixture was stirred for 1 hr. After that, more water (200 ml) was added, and the mixture was extracted with ether (three times). The aqueous layer was carefully acidified to pH 3 with dilute HCl (1 M) while stirring in

an ice bath, and extracted with ether (three times). The combined ether extracts were washed with water and dried over anhydrous sodium sulfate. After evaporation of the solvent, the material was pumped under vacuum, and crude crystals of the hydroxycarboxylic acid (9.8 g) were obtained. This crude hydroxycarboxylic acid was dissolved in dry ether (20 ml), and dry hexane (120 ml) was added to the solution. The solution was kept standing overnight at room temperature. Crystals (8 g) were isolated by filtration. Pure hydroxycarboxylic acid (more than 99.6% ee) (5 g) was obtained by repeating this procedure twice. A solution of pure hydroxycarboxylic acid (5 g) and *p*-toluenesulfonic acid dissolved in ether (30 ml) was refluxed for 1 hr. After cooling, the solution was washed with water and aqueous sodium carbonate and dried over anhydrous magnesium sulfate. Chemically and enantiomerically pure buibuilactone (4.4 g) was obtained. The same procedure was followed for the preparation of chemically and enantiomerically pure (*S*)-buibuilactone and (*R*)- and (*S*)-japonilure. The optical purity of these semiochemicals was determined to be >99.6% by chiral GC (Leal, 1991). Synthetic chemicals (1 and 5 mg for *A. octiescostata* and *A. cuprea*, respectively) were incorporated in plastic pellets (4–5 mm in diameter) made of a polyethylene–vinyl acetate. The pellets were placed inside pellet holder (Fuji Flavor Co., Tokyo) and set in the traps.

Field Tests and Indoor Bioassays. Field tests were conducted in the Tsukuba area either in golf courses (*A. octiescostata*) or chestnut fields (*A. cuprea*) utilizing Windspack traps (Japan Tobacco, Tokyo) set with the pheromone dispenser ca. 30 cm and 1.5 m above the soil for *A. octiescostata* and *A. cuprea*, respectively. The traps were replicated in randomized blocks with the intertrap distance of 10 m. Capture data were transformed to log $(x + 1)$ before differences between means were tested for significance by ANOVA with JMP software (Anonymous, 1989). In the figures, treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test. Means of captures are untransformed, and error bars show one standard error (SE). Behavioral responses of male beetles were also observed in a glass Y-olfactometer (long arm, 27 cm; side arms, 21 cm; 7.5 cm ID; airflow, 300 ml/min; relative humidity 58%, 25°C) during the photophase. A test sample (5 μl × 100 ng/μl) was loaded on a piece of filter paper (1 × 1 cm); the control was loaded with an equal amount of hexane. After evaporation of the solvent (ca. 30 sec) each filter paper was placed in one of the side arms and the beetle behavior was observed for 15 min. Clean air was blown through the olfactometer for 10–15 min between tests, and the treatment and control sides were reversed. These data were analyzed by the Wilcoxon/Kruskal-Wallis test with a JMP software (Anonymous, 1989).

Chiral Discrimination by the Human Nose. Enantiomerically pure samples of the enantiomers of buibuilactone and japonilure were analyzed by two independent panels of specialists (Fuji Flavor Co. Ltd., Tokyo, and Takasago International Co., Hiratsuka-city, Kanagawa Prefecture). The odor quality and strength

of the compounds dissolved in hexane in five-step dilutions (1000, 100, 10, 1, and 0.1 ppm) was evaluated by smelling a slender piece of paper soaked in the test solutions (blotter test). The Fuji Flavor and Tasakago panels had three members and one perfumer, respectively.

RESULTS AND DISCUSSION

The sex pheromone system for *A. octiescostata* has been identified to be a 4 : 1 binary mixture of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one [(*R*)-buibuilactone] and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one [(*R*)-japonilure] (Figure 1) (Leal et al., 1994). The stereochemistry of the major constituent, buibuilactone, was determined by comparison of synthetic standards with the natural product on gas chromatography using a chiral column. The lactone isolated from the airborne volatiles of female *A. octiescostata* gave the same retention time as (*R*)-buibuilactone and no traces of the *S* stereoisomer were found in the natural sample. The absolute configuration of the minor constituent (japonilure) was inferred, but the scanty amount of natural material prevented its analytical characterization (Leal et al., 1994). The effect of the nonnatural stereoisomers on the pheromonal activity was not addressed because a synthetic mixture of enantiomerically pure (*R*)-lactones was highly attractive in the field. However, the recent discovery of the mutual agonist–antagonist activities of enantiomeric pheromones in two scarab species (Leal, 1996) motivated further investigation of the effect of chirality on pheromonal activity.

First, I compared the responses of males to traps loaded with a binary mixture [(*R*)-buibuilactone plus (*R*)-japonilure] with those baited with a single (major or minor) constituent. Traps baited with the binary mixture had the best performance, catching over 400 beetles per trap per day (Figure 2). Although captures in traps baited with the minor component (*R*)-japonilure were not significantly higher than those in control traps (data not shown), the major pheromone constituent [(*R*)-buibuilactone] alone captured significantly more beetles than control traps (Figure 2). These findings indicate that (*R*)-japonilure is essential to achieve the best performance in captures of *A. octiescostata*.

The effect of chirality on pheromonal activity was tested in the field by comparing the captures in traps baited with enantiomerically pure samples of both constituents to those in traps baited with racemic pheromones, either major or minor constituent (the other component being enantiomerically pure). There was no significant difference in catches in traps baited with the (1) minor constituent as a racemic mixture, (2) the major constituent as a racemic mixture, and (3) enantiomerically pure pheromones (Figure 3). Thus, (*S*)-buibuilactone and (*S*)-japonilure are neither attractants nor behavioral antagonists for pheromonal activity in *A. octiescostata*.

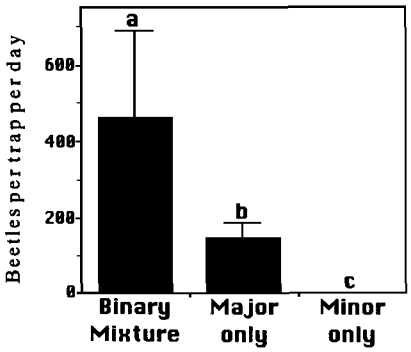

FIG. 2. Trap catches of *A. octiescostata* in traps baited with single- and two-component lures. Catches by the minor component, (*R*)-japonilure, were not significantly different from those in control (unbaited) traps. Captures by the major component, (*R*)-buibuilactone, was significantly higher than in control traps, whereas the best performance was obtained with the two components (binary mixture).

In order to investigate whether the antennae of *A. octiescostata* males possess ORNs tuned to both enantiomers of buibuilactone, I utilized a previously reported chiral GC-EAD system (Leal, 1996), which takes advantage of the power of chiral resolution to expose an antennal preparation to enantiomers completely devoid of their antipodes. In the chiral GC-EAD, male antennae of *A. octiescostata* responded only to (*R*)-buibuilactone (Figure 4), whereas the non-

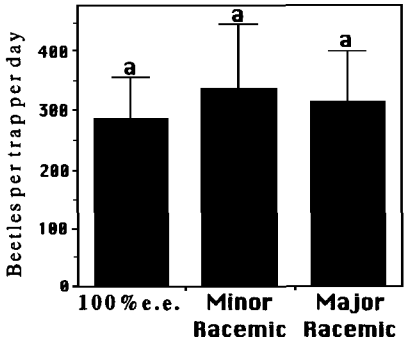

FIG. 3. Response of *A. octiescostata* to traps baited with enantiomerically pure buibuilactone and japonilure (100% ee), or using one constituent enantiomerically pure and the other as a racemic mixture. Captures in traps baited with enantiomerically pure pheromone were not significantly different from those having the minor or the major constituents [(*R*)-japonilure and (*R*)-buibuilactone, respectively] as a racemic mixture.

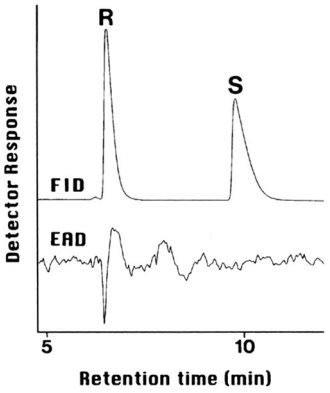

FIG. 4. Enantiomeric resolution of (*R*)- and (*S*)-japonilure on a chiral capillary column and parallel EAD response of excised male antennae of *A. octiescostata*.

natural *S* stereoisomer remained undetected. If the antennae had detectors for both enantiomers, one would expect to get peaks of similar shapes (possibly with different intensities) related to both stereoisomers (Leal, 1996). In order to rule out that the possibility that the lack of response to the *S* enantiomer was due to sensory adaptation of the antennae, the racemic mixture was injected twice so that the first peak of the second injection coeluted with the second peak of the first injection. In doing so, the antennal preparations were exposed to both *R* and *S* stereoisomers at the same time [after it has been stimulated with the natural (*R*)-buibuilactone]. The recordings clearly show that the antennae also responded to the second peak when it contained the *R* enantiomer (Figure 5). These findings indicate that male antennae of *A. octiescostata* do not possess ORNs that can detect the nonnatural enantiomer, (*S*)-buibuilactone. This "enantiomeric anosmia"—the inability to "smell" the antipode of its pheromone—in this species is remarkably different from what has been observed in the Japanese and Osaka beetles (Leal, 1996).

Scarab beetles possess lamellar antennae, formed by leaflike extensions of the terminal segments, on which the olfactory sensilla are located. Using single sensillum recordings, it has been demonstrated that pheromone-sensitive receptor neurons are located in sensilla placodea without pits (Leal and Mochizuki, 1993), which encompasses a large area of the posterior part of the lamellae. In *A. cuprea*, these receptors are specific to (*R*)-buibuilactone, whereas its antipode remains undetected (Leal and Mochizuki, 1993). Although the occurrence of *S*-specific ORNs in *A. cuprea* could not be demonstrated, their existence was hypothesized (Leal and Mochizuki, 1993) based on an inhibitory effect of (*S*)-buibuilactone observed in a walking bioassay and an EAG response. In order

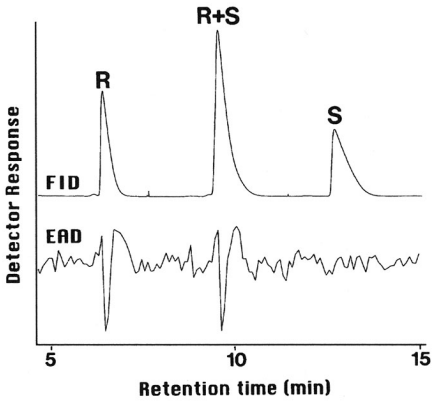

FIG. 5. Parallel FID and EAD chromatograms obtained by the injection of two racemic mixtures of buibuilactone so that the *R* peak from the second injection coeluted with the *S* peak of the first injection. A male *A. octiescostata* antenna was used as the sensing element.

to test this hypothesis, I used chiral GC-EAD. Surprisingly, male antennae of *A. cuprea* gave no detectable response to the nonnatural *S* stereoisomer when it is completely devoid of the natural enantiomer, although they clearly responded to (*R*)-buibuilactone (Figure 6). As in the case of *A. octiescostata* (Figure 5), male antennae of *A. cuprea* responded to the *R* enantiomer when it was made to

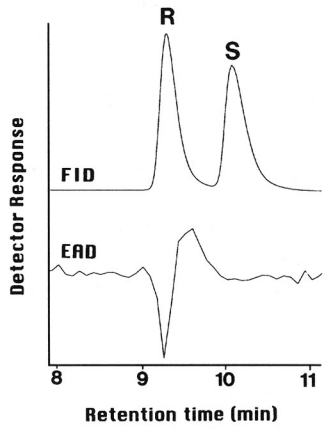

FIG. 6. Simultaneous responses of FID and EAD (antenna of male *A. cuprea*) to a racemic mixture of buibuilactone separated on a chiral capillary column. Note that despite the baseline separation, the column has lower performance than in the previous experiments.

coelute with the *S* stereoisomer (data not shown), thus ruling out the possibility of sensory adaptation. These findings indicate that male antennae of *A. cuprea* do not possess detectors for the *S* enantiomer. Further investigations by tungsten penetration technique (single sensillum recordings) corroborated the lack of (*S*)-buibuilactone-sensitive ORNs in *A. cuprea* (Larsson et al., 1999).

Considering the possibility that previous behavioral experiments in a plastic box under still air (Leal and Mochizuki, 1993) could be misleading, and in light of the new findings regarding ORNs, I reexamined the effect of chirality on the attraction of *A. cuprea* males using a different bioassay. In a Y-type olfactometer, males of *A. cuprea* were attracted only to the pheromone source and I never observed a single beetle being attracted to the control side arm (N = 25). Thus, the behavioral bioassay in a glass Y-olfactometer seems to be more reliable than the previous one (Leal and Mochizuki, 1993). To determine the effect of (*S*)-buibuilactone on the male attraction, I carried out a series of observations, in which the attraction of one group of males was tested (1) against enantiomerically pure samples of (*R*)-buibuilactone plus (*R*)-japonilure vs. hexane (control) and (2) a mixture of racemic buibuilactone plus (*R*)-japonilure vs. hexane. Eight males were released in the arena and observed for 15 min with the choices of hexane versus the racemic buibuilactone [plus (*R*)-japonilure] and in a different run hexane versus (*R*)-buibuilactone plus (*R*)-japonilure. The first test (racemic or enantiomerically pure) was randomly selected and the other choice was repeated soon after cleaning the arena with pure air. These experiments clearly showed that the nonnatural (*S*)-buibuilactone was not a behavioral antagonist (Figure 7). A possible explanation for the previous observations that male response decreased when the pheromone sample was spiked with the nonnatural

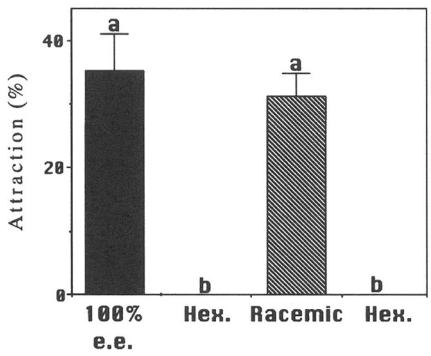

FIG. 7. Behavioral response of male *A. cuprea* in an olfactometer to (*R*)-buibuilactone plus (*R*)-japonilure and to a racemic mixture of buibuilactone plus enantiomerically pure japonilure.

(*S*)-buibuilactone (Leal and Mochizuki, 1993) is that the effect was due to the high dosages of the pheromone (>5× higher than in the olfactometer observations) and to adsorption of the test compounds on the wall of the plastic cages. I was unable to reproduce the previous results using similar cages and testing beetles collected in Ibaraki and Chiba prefectures (the previous experiments were conducted with insects collected in the Kyushu area, southern Japan). In addition, the observation in the olfactometer is more reliable statistically due to a larger sample ($N = 25$) than that in the previous experiments ($N = 5$). Preliminary field tests in Tsukuba further support the conclusion that (*S*)-buibuilactone is not a behavioral antagonist. Catches in traps baited with enantiomerically pure samples of the pheromone were not significantly different from those in traps loaded with racemic buibuilactone plus (*R*)-japonilure.

During the preparation of samples for these experiments I observed that the enantiomers of buibuilactone and japonilure smell different. In a fragrance evaluation by two independent panels of skilled perfumers, (*S*)-japonilure was described as having a strong lactonelike and medicinal odor, somewhat citrus aldehydic, whereas the *R* enantiomer was classified as a metallic lactonelike green odor. The *S* form was perceived at 10–100 times lower concentrations than the *R* stereoisomer, which had a 1000 ng/μl (=1 μg/μl) threshold. On the other hand, the odor intensity of the enantiomers of buibuilactone, a lactone shorter by two carbons, was reversed. While (*R*)-buibuilactone was classified as much stronger balsamic, sweet, and fruit odor, in the direction of pineapple with a fatty note, (*S*)-buibuilactone was described as fruity, slightly heavy and ripe, in the direction of peach. The thresholds for (*R*)- and (*S*)-buibuilactone were 10 and 100–1000 ng/μl, respectively.

Although the human nose can detect both enantiomers of the two lactones, male antennae of *A. octiescostata* and *A. cuprea* are "blind" to the nonnatural (*S*)-buibuilactone. This enantiomeric anosmia seems to be a common feature in scarab beetles. We have previously observed that there was no significant difference in catches of the large black beetle, *Holotrichia parallela*, in traps baited either with enantiomerically pure L-isoleucine methyl ester plus (*R*)-linalool or a random mixture of stereoisomers plus (*R*)-linalool. In addition, catches in traps baited with the major constituent plus (*R*)-linalool were significantly higher than captures with L-isoleucine methyl ester alone. Furthermore, the addition of (*S*)-linalool to the complete mixture did not decrease the activity of the pheromone (Matsuyama et al., 1994). Likewise, preliminary field experiments suggested that catches of the yellow elongate chafer, *Heptophylla picea*, with its synthetic pheromone, (*R,Z*)-7,15-hexadecadien-4-olide (Leal et al., 1996), was not significantly different from captures in traps baited with a racemic mixture of the lactone (Leal, Kuwahara, and Mori, unpublished data).

The major sex pheromone components for the cranberry white grub, *Phyllophaga anxia*, has been identified as L-valine and L-isoleucine methyl esters

(Zhang et al., 1997). In chiral GC-EAD experiments, L-valine methyl ester elicited strong antennal responses, whereas the D-enantiomer was EAD inactive. On the other hand, D-isoleucine methyl ester and D-*allo*-isoleucine methyl ester diastereomers were EAD inactive, but both L-isoleucine methyl ester and L-*allo*-isoleucine methyl ester diastereomers elicited equally high EAD responses (Zhang et al., 1997). It may be the case that the antennae of the cranberry white grub do not possess ORNs tuned to the nonnatural stereoisomers of both pheromone constituents; the smaller response due to L-*allo*-isoleucine methyl ester may be due to nonspecific activation of the L-isoleucine methyl ester receptors.

In conclusion, the inability of scarab beetles to detect nonnatural stereoisomers of their chiral sex pheromones seems to be a common feature, whereas the previously identified case of mutual behavioral agonist–antagonist of enantiomeric pheromones in the Japanese beetle and Osaka beetle is more an exception than a rule. These findings shed more light on the evolutionary significance of the mutual ability to detect the allospecific pheromones in *P. japonica* and *A. osakana*. Further investigations will clarify whether the mutual behavioral antagonism is a remnant of past interaction among scarab beetles.

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SEX ATTRACTANT PHEROMONE OF SATURNIID MOTH *Coloradia velda*

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Abstract—The female-produced sex attractant pheromone of *Coloradia velda* has been identified by a combination of analytical methods and field tests as a blend of (10*E*,12*Z*)-hexadecadien-1-yl acetate (10*E*,12*Z*–16 : Ac), (10*E*,12*E*)-hexadecadien-1-yl acetate (10*E*,12*E*–16 : Ac), and (10*E*,12*Z*)-hexadecadien-1-ol (10*E*,12*Z*–16 : OH) in a 10 : 1 : 0.33 ratio, respectively. The corresponding aldehyde (10*E*,12*Z*–16 : Ald) was also found in extracts of female pheromone glands, but in blends with 10*E*,12*Z*–16 : Ac and 10*E*,12*E*–16 : Ac at low doses did not significantly increase the attractiveness of baits to male moths.

Key Words—Pheromone, *Coloradia velda*, *Coloradia doris*, Saturniidae, (10*E*,12*Z*)-hexadecadienyl acetate, (10*E*,12*E*)-hexadecadienyl acetate, (10*E*,12*Z*)-hexadecadienol, (10*E*,12*Z*)-hexadecadienal.

INTRODUCTION

Coloradia velda Johnson & Walter (Lepidoptera: Saturniidae) is a large, cryptically colored moth whose known range is restricted to the San Bernardino mountains of southern California (Tuskes et al., 1996). It originally was thought to be a geographic variant of *C. doris*, but in 1979 Johnson and Walter (1979) reclassified it as a distinct species. It occurs in the juniper–pinyon woodland from 1000 to 2700 m. Its principal larval host appears to be pinyon pine, *Pinus monophylla* Torrey & Fremont (Tuskes et al., 1996). The life history has been described by Tuskes (1984). Eggs are laid in clusters on the host, and the first through third instars are gregarious while the fourth and fifth instars are solitary feeders. The

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larvae pupate in August and diapause in loosely spun cocoons just below the soil surface, emerging as adults the following May and June. Males tend to fly early in the evening from dusk to about 22 : 00 hr, while females fly until well past midnight. The species is univoltine.

In conjunction with studies of other saturniid moths in the genus *Coloradia* Blake, and in the related genus *Hemileuca* Walker, we became interested in examining the pheromone chemistry of *C. velda*. We report here the identification and field testing of components of the female-produced sex attractant pheromone of this species.

METHODS AND MATERIALS

Insects. In May and June of 1995, 1996, and 1997, male and female moths responding to UV-light traps were collected at Cactus Flats in the northeastern San Bernardino Mountains, San Bernardino County, California. Females were allowed to oviposit on pinyon branches, while males were held in glassine envelopes at 4–15°C until required for experiments. Neonate larvae were reared on bouquets of pinyon pine branches in screen-covered 40-liter glass aquaria, changing bouquets when the larvae had consumed the foliage. Once feeding ceased, wandering larvae were placed in plastic boxes containing peat moss to pupate. Pupae were held outdoors in a screen cage in a shaded area until emergence. Newly emerged females were held in screen cages to permit them to call, while males were held at 4–15°C until needed. Refrigeration was necessary because of the short life-span of the nonfeeding adults.

Preparation and Analysis of Pheromone Extracts. The ovipositor was extruded by gentle pressure on the abdomen and the terminal abdominal segments, including the pheromone gland, were clipped from laboratory-reared, calling female moths (0–2 days old) from 1 to 2 hr after sunset. The excised tips were extracted in 30–50 μl pentane for 10 min, and then the extracts were transferred to clean vials with a single 20-μl rinse. Extracts were stored in the freezer at −20°C until needed for coupled gas chromatographic/electroantennographic or chemical studies.

Coupled Gas Chromatography and Electroantennography. Extracts and synthetic standards (1 ng in 1 μl heptane) were analyzed by coupled gas chromatography–electroantennogram detection (GC-EAD), using a Hewlett-Packard 5890 series II GC (Hewlett-Packard, Palo Alto, California) and a custom-built amplifier as previously described (McElfresh and Millar, 1999a). The GC was operated in splitless mode and was equipped with a DB-5 column (30 m × 0.32 mm ID, 0.25-μm film thickness; J&W Scientific, Folsom, California) programmed from 100°C for 0 min, 10°/min to 275°C/20 min, or a DB-1701 column (30 m × 0.32 mm ID, 0.25-μm film) with a program of 100°C for 0 min, 10°/min to

275°C/10 min, or an HP Ultra-1 column (30 m × 0.25 mm ID, 0.17-μm film) with a program of 100°C for 1 min, 5°/min to 210°C/0 min, 20°C/min to 300°C/30 min. Effluent from the column was split between two 1-m sections of deactivated fused silica tubing (0.25 mm ID). One branch was directed to the flame ionization detector (FID) while the other branch was directed through a heated conduit (250°C) into an ambient temperature humidified airstream (~500 ml/min), which passed over the antennal preparation. Male antennae were detached from the head by pulling them off with forceps, the terminal rami and segments were removed, and the antennae were mounted between two saline-filled (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1-l distilled water) glass capillary electrodes, with chloridized silver wires down the center of each electrode. GC and EAD traces were recorded simultaneously on a matched pair of H-P 3394 recording integrators.

Coupled Gas Chromatography-Mass Spectrometry. Extracts were analyzed by coupled GC-mass spectrometry (GC-MS), using an HP 6890 GC interfaced to an HP 5973 mass selective detector (electron impact ionization, 70 eV). The GC was equipped with an HP5-MS column (30 m × 0.25 mm ID, 0.25-μm film thickness), programmed from 50°C/1 min, 10°/min to 250°C, operated in splitless mode. A computerized database (NIST/EPA/NIH, 1992) was used to assist with preliminary identification of compounds. Identifications were then verified by comparison of GC retention times on all columns and mass spectra of insect-produced compounds with those of synthetic standards (see below).

An insect extract was also derivatized with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD, Aldrich Chemical, Milwaukee, Wisconsin), which forms Diels-Alder adducts with conjugated dienes, to locate the positions of the double bonds in the diene components of the pheromone (Young et al., 1990; McElfresh and Millar, 1999a). An aliquot of a pheromone gland extract (~1/2 female equivalent) was concentrated under N_2, then taken up in 20 μl CH_2Cl_2. To this solution was added 2.5 μl of MTAD in CH_2Cl_2 (1.2 mg/ml). The resulting solution was concentrated to <5 μl, and a 1-μl aliquot was analyzed by GC-MS using the HP5-MS column, using an injector temperature of 300°C, and a temperature program of 100°C for 0 min, 15°/min to 300°C for 20 min, and a solvent delay time of 8 min. Identifications of derivatized compounds were confirmed by analyses of adducts prepared in identical fashion from synthetic standards.

Gas Chromatography. In addition to the above columns used in coupled GC-EAD, a DB-17 column (30 m × 0.32 mm ID, 0.25-μm film; program 100°C/0 min, 10°/min to 275°C/10 min) in a Hewlett-Packard 5890 series II GC was used for further confirmation of the 10*E*,12*Z*–16 : Ac identity. Identification of other components on this column was not possible due to coelution problems.

Synthetic Standards. Synthetic standards of the four 10,12–16 : Ac isomers, 10*E*,12*Z*–16 : OH, and 10*E*,12*Z*–16 : Ald were prepared as previously

described (McElfresh and Miller, 1999a). Hexadecanol and hexadecyl acetate were obtained from Aldrich Chemical.

Field Trials. Field trials were conducted east of Big Bear City in the San Bernardino Mountains, San Bernardino County, California, from late May to late June 1997 and 1998. Baits consisted of heptane solutions of test compounds, at semilog rates from 0 to 100 μg/compound/bait, loaded onto 11-mm red rubber septa (Thomas Scientific, Philadelphia, Pennsylvania) with two drops of a solution of butylated hydroxytoluene (BHT) in heptane (10 mg/ml) added as antioxidant. Loaded baits were impaled on fish hooks for ease of handling and to permit suspension inside traps. All lures were stored in 20 ml vials in a freezer (-20°C) prior to use, and no lures were used for more than three days. Pherocon 1C traps (Trécé Inc., Salinas, California) were used but were modified by addition of a wire in the center of the lid which allowed suspension of the lure. Individual traps were placed from 1 to 2 m high in pinyon or juniper trees at approximately 10 m apart in a line. A field trial consisted of 5–10 replicate blocks located at least 0.5 km apart. Trials were set up and trap catches were tabulated in the late afternoon prior to initiation of flight by males. Trap catches were tabulated at one to two-day intervals, depending upon weather conditions.

Statistical Analysis. Because the day effect was not relevant to the trials, it was eliminated by pooling the day counts for each treatment in a given block. This sum was then transformed ($\sqrt{x} + 0.5$) prior to conducting a two-way ANOVA, followed by linear and quadratic contrasts. If significant effects were detected, then Student-Neuman-Keuls (SNK, $\alpha = 0.05$) test was used to separate means (SAS Institute, 1996).

RESULTS

Identification of Pheromone Components. In studies with several species of saturniid moths in the subfamily Hemileucinae, 10,12-hexadecadienyl acetates, aldehydes, and alcohols have been shown to be sex attractant pheromone components for several species (McElfresh and Millar, 1999a,b; unpublished data). Consequently, our first experiment with *C. velda* involved testing GC-EAD responses of antennae from several male moths (captured at UV lights) to a series of synthetic standards, including 10*E*,12*Z*–16 : Ac, 10*E*,12*E*–16 : Ac, 10*E*,12*Z*–16 : OH, 10*E*,12*Z*–16 : Ald, 16 : Ac, and 16 : OH. The antennae responded strongly to 10*E*,12*Z*–16 : Ac, with smaller responses to 10*E*,12*Z*–16 : OH (26.3 ± 1.5% of response to 10*E*,12*Z*–16 : Ac; $N = 3$) and 10*E*,12*Z*–16 : Ald (23.0 ± 3% of response to 10*E*,12*Z*–16 : Ac; $N = 3$). Analogous results were obtained in 1997 and 1998 (10*E*,12*Z*–16 : OH response, 36.3 ± 4.8% of response to 10*E*,12*Z*–16 : Ac, $N = 7$; 10*E*,12*Z*–16 : Ald, 23.0 ± 2.9% of response to 10*E*,12*Z*–16 : Ac, $N = 7$). In addition, 16 : Ac also elicited weak

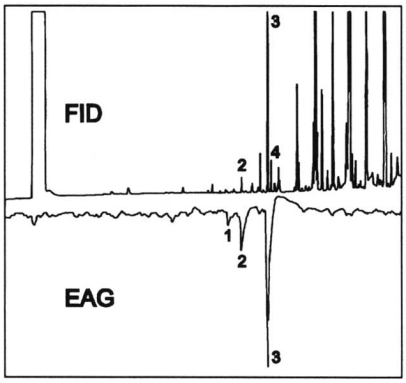

FIG. 1. Coupled gas chromatogram–electroantennogram of male *Coloradia velda* antenna stimulated by a female extract. Column: DB-5 (30 m × 0.32 mm ID, 0.25-μm film; program 100°C for 0 min, 10°C/min to 275°C, hold for 20 min). Identifications of peaks: (1) 10*E*,12*Z*–16 : Ald, (2) 10*E*,12*Z*–16 : OH, (3) 10*E*,12*Z*–16 : Ac, and (4) 10*E*,12*E*–16 : Ac.

antennal responses (4.6 ± 2.4% of response to 10*E*,12*Z*–16 : Ac, $N = 6$). Male antennae also responded to 10*E*,12*E*–16 : Ac but this response could not be accurately quantified because the EAD peaks from the 10*E*,12*Z* and 10*E*,12*E* isomers were not resolved. There was no response to 16 : OH.

In 1996 through 1998, pheromone gland extracts from laboratory-reared female moths were analyzed by GC-EAD (Figure 1, Table 1). In all of the 48 single female extracts examined in 1997 and 1998, male moth antennae exhibited strong responses to a peak eluting at the retention time of 10*E*,12*Z*–16 : Ac, with smaller but consistent responses to a peak eluting at the retention time of

TABLE 1. ELECTROANTENNOGRAPHIC RESPONSES RELATIVE TO THAT OF 10*E*,12*Z*–16 : Ac, AND PERCENTAGE OF MALE *Coloradia velda* ANTENNAE DETECTING INDIVIDUAL COMPOUNDS IN GC-EAD ANALYSES OF FEMALE EXTRACTS[a]

Compound	Size of response		% detected
	Mean (%)	Range (%)	
10*E*,12*Z*-16 : Ac	100		100
10*E*,12*Z*-16 : OH	12	6–20	87
10*E*,12*Z*-16 : Ald	3.5	1.7–8.6	45
10*E*,12*E*-16 : Ac	18	16–21	13
16 : Ac	2.3	1.4–3.3	94

[a]Responses from 10 males, 6 in 1997, 4 in 1998 to a total of 38 female extracts.

TABLE 2. COMPOUNDS, METHODS OF IDENTIFICATION, AND AMOUNTS RELATIVE TO 10*E*,12*Z*–16 : Ac IN EXTRACTS OF FEMALE *Coloradia velda* FROM THE SAN BERNARDINO MOUNTAINS

	Identification methods used[a]			Amount in single female extracts relative to 10*E*,12*Z*–16 : Ac[b]			
Compound	GC	GC-MS	EAD	Mean	SD	Range	% detected[c]
10*E*,12*Z*–16 : Ac	X	X	X	100			100
10*E*,12*Z*–16 : OH	X	X	X	69	53	17–177	37
10*E*,12*Z*–16 : Ald			X				0
10*E*,12*E*–16 : Ac	X	X	[d]	17	10	9–56	56
16 : Ac	X	X		46	29	13–110	100

[a]GC = retention time matches on at least three columns (see Methods and Materials) with Authentic standards. GC-MS = mass spectral match with authentic standards. EAD = antennal response at retention time of authentic standard.
[b]Means of 41 of 48 female extracts which possessed measurable amounts of 10*E*,12*Z*–16 : Ac.
[c]Percent of female extracts with FID-detectable amounts of individual compounds.
[d]EAD signal was not resolved from that of 10*E*,12*Z*–16 : Ac.

10*E*,12*Z*–16 : OH. A small and less consistent response was seen at the retention time of 10*E*,12*Z*–16 : Ald in the EAD trace, although no peak could be seen in the FID trace (Figure 1).

The identities and relative amounts of compounds in the pheromone gland extracts were confirmed by GC on multiple columns and GC-MS (Table 2). The retention times of the compound eliciting the strongest response from male antennae matched those of a 10*E*,12*Z*–16 : Ac standard on four GC columns of different polarity (DB-17, DB-5, HP Ultra 1, DB-1701), and the mass spectrum matched that of the standard (molecular ion, m/z 280, base peak, m/z 67). The conjugated diene was confirmed to be in the 10,12 position by examination of the mass spectrum of the Diels-Alder adduct formed between 10*E*,12*Z*–16 : Ac and the dienophile MTAD (Young et al., 1990; McElfresh and Millar, 1999a). The adduct exhibited a molecular ion at m/z 393 (6%), and diagnostic fragments at m/z 350 (23%) and 208 (100%) from cleavage of the alkyl chains attached to the carbons α to the nitrogens in the six-membered ring of the adduct (Figure 2). Confirmation of the stereochemistry of the diene was obtained from comparison of the retention time of the underivatized insect-produced compound on a DB-5 column with those of synthetic standards of the four 10,12–16 : Ac isomers (McElfresh and Millar, 1999a,b). Only the 10*E*,12*Z*–16 : Ac isomer had the same retention time as the insect-produced compound. The mean estimated amount of 10*E*,12*Z*–16 : Ac was ~1.5 ng/female extract, with the maximum seen being ~5.5 ng/extract.

10*E*,12*Z*–16 : OH was identified in the insect extracts in similar fashion. The insect-produced compound matched the retention times of 10*E*,12*Z*–16 : OH on

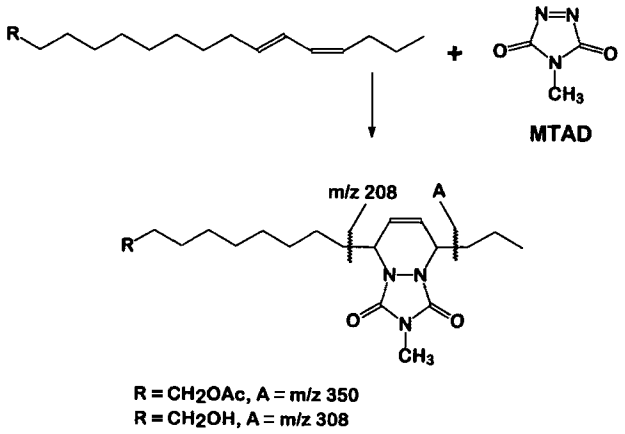

FIG. 2. Formation of Diels-Alder adducts of the conjugated diene pheromone components with MTAD, and the resulting cleavages giving diagnostic fragment ions in the EI mass spectra of the derivatives.

three columns (DB-5, HP Ultra 1, DB-1701), but it was not possible to obtain a full-scan mass spectrum due to the small amount of material in the extract and coeluting compounds in the extract analyzed. However, derivatization with MTAD shifted the peak to a clear region of the chromatogram, providing a full scan mass spectrum of the derivative. Specifically, the MTAD adduct showed a molecular ion at m/z 351 (17%), and diagnostic ions at m/z 308 (50%) and 208 (100%) from cleavages of the alkyl chains from the heterocyclic ring (Figure 2). The retention time and mass spectrum of the adduct also matched that of the adduct prepared from an authentic sample of 10*E*,12*Z*–16 : OH. The stereochemistry of the dienol was unambiguously determined by comparison of the retention times of the insect-produced compound with samples of the four 10,12–16 : OH isomers on DB-5 and HP Ultra 1 columns; only the 10*E*,12*Z*–16 : OH isomer produced an exact match.

The identification of 10*E*,12*Z*–16 : Ald remains tentative because it was not possible to obtain a mass spectrum of the aldehyde or its MTAD adduct, nor was an FID response observable on any column due to the very small amounts of this compound in extracts. The evidence we have for its presence is the fairly consistent EAD response at the retention time for 10*E*,12*Z*–16 : Ald on three different columns (DB-5, 14 of 28 with *C. velda* males; HP Ultra-1, 3 of 10 extracts; DB-1701, 1 of 2 extracts). Since 10*E*,12*Z*-16 : Ald is a known pheromone component for several related species [*Hemileuca* spp. (McElfresh and Millar, 1999a,b), and at least one other *Coloradia* species (McElfresh and Millar, unpublished data)], it was included in field trials.

In addition to the major antennal stimulatory compounds, several other components of the pheromone gland extract were identified by matchup of mass spectra and GC retention times on an HP5-MS column with those of authentic standards. These included 16 : Ac, 20 : OH, C_{21}–C_{24} unbranched alkanes, and 10*E*,12*E*–16 : Ac. The identity of the latter compound was further confirmed by retention time matches on three other columns of differing polarity (DB-17, DB-1701, H-P Ultra-1).

Field Trials. In the first field trial testing blends of 10*E*,12*Z*–16 : Ac (contaminated with 3.8% of the 10*E*,12*E*–16 : Ac isomer) with 10*E*,12*Z*–16 : OH, the blend containing 0.33% of the alcohol attracted the most moths (Figure 3A), although it was not statistically better than the 1% rate. Rates of 3.3% and above captured significantly fewer moths, as did rates of 0.1% or below. The bait containing no 10*E*,12*Z*–16 : OH was still reasonably attractive, indicating that this compound enhances but is not essential for attraction.

A second field trial tested the single component 10*E*,12*Z*–16 : Ac versus blends of 10*E*,12*Z*–16 : Ac with 10*E*,12*Z*–16 : Ald (Figure 3B). Addition of 0.01 and 0.1 μg 10*E*,12*Z*–16 : Ald had no effect on the trap captures, but the 1- and 10-μg doses resulted in significant decreases in trap captures relative to 10*E*,12*Z*–16 : Ac alone.

A third field trial was conducted to assess the role of 10*E*,12*E*–16 : Ac more carefully. In previous years, the 10*E*,12*Z*–16 : Ac used to make lures contained 3.8% of this isomer as a by-product of its synthesis. Using argentized ion exchange chromatography (Houx et al., 1974) samples of the 10*E*,12*Z*–16 : Ac were purified to 98.4% purity. To this, various rates of 10*E*,12*E*–16 : Ac were then added. The results of the field trial indicate that this isomer is an important part of the blend, with the 35% and 12% rates attracting significantly more males than either the 100% or the 5% or lower rates (Figure 3C).

The final field trial compared the complete blend of all three components to blends lacking one of those components (Figure 4). Elimination of 10*E*,12*Z*–16 : Ac resulted in no trap catches, confirming its importance as the major component. Elimination of either 10*E*,12*Z*–16 : OH or 10*E*,12*E*–16 : Ac resulted in significant reductions in trap catches, confirming that they are indeed important parts of the blend.

DISCUSSION

Prior to 1998, 10*E*,12*Z*–16 : Ac [the acetate of 10*E*,12*Z*–16 : OH or bombykol, the first insect pheromone identified, reviewed in Hecker and Butenandt (1984)], had been reported as a sex pheromone component from only a single moth species, *Rondotia menciana* Moore (Bombycidae) (Dai et al., 1987). We now have identified this compound as the major component of the sex

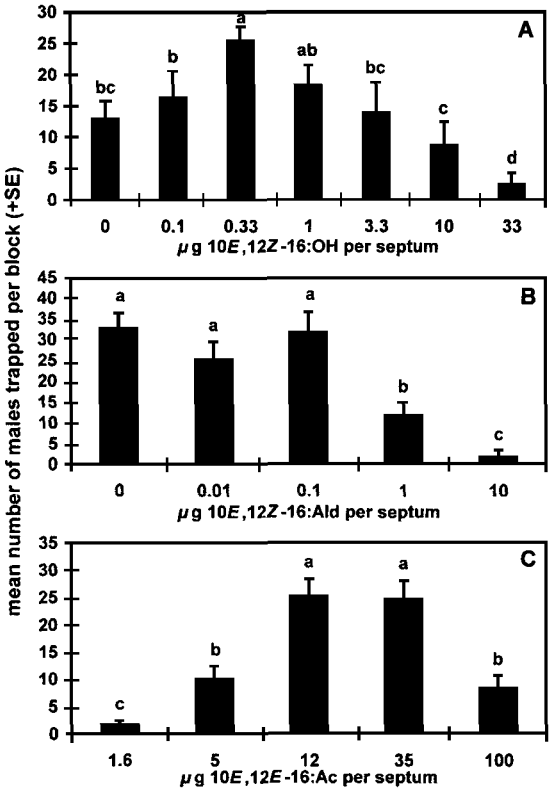

FIG. 3. (A) Results of *Coloradia velda* field trials (June 6–8, 1997) with 10*E*,12*Z*–16 : Ac and 10*E*,12*E*–16 : Ac held constant at 96 and 4 μg/septum, respectively, and variable rates of 10*E*,12*Z*–16 : OH. Total number of moths trapped was 485. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for 10*E*,12*Z*–16 : OH rate effect, $F = 16.9$, $df = 6, 24$, $P < 0.001$; linear contrast $F = 47.6$, $df = 1, 24$, $P < 0.001$, quadratic contrast $F = 49.7$, $df = 1, 24$, $P < 0.001$. (B) Results of *Coloradia velda* field trial (June 10–12, 1997) for optimization of the 10*E*,12*Z*–16 : Ald rate, with 10*E*,12*Z*–16 : Ac and 10*E*,12*E*–16 : Ac held constant at 96 and 4 μg/septum, respectively. Total number of moths trapped was 463. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for 10*E*,12*Z*–16 : Ald rate effect, $F = 18.5$, $df = 4, 16$, $P < 0.001$; linear contrast $F = 57.6$, $df = 1, 16$, $P < 0.001$, quadratic contrast $F = 11.4$, $df = 1, 16$, $P = 0.0039$. (C) Results of *Coloradia velda* field trial (June 15–16, 1998) for optimization of the 10*E*,12*E*–16 : Ac rate, with 10*E*,12*Z*–16 : Ac held constant at 100 μg/septum. Total number of moths trapped was 352. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two way ANOVA for 10*E*,12*Z*–16 : Ald rate effect, $F = 44.9$, $df = 4, 16$, $P < 0.001$; linear contrast $F = 40.1$, $df = 1, 16$, $P < 0.001$, quadratic contrast $F = 129.6$, $df = 1, 16$, $P < 0.001$.

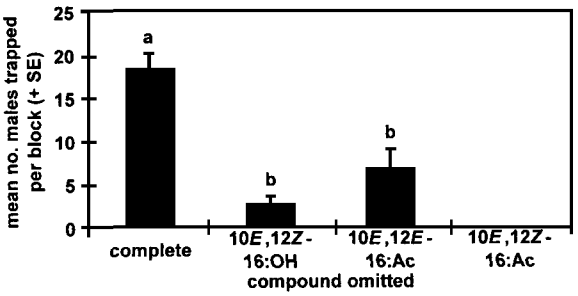

FIG. 4. Attractiveness to male *Coloradia velda* of blends containing single omissions from the complete blend. Amounts of materials (μg/septum): 10*E*,12*Z*–16:Ac, 100; 10*E*,12*Z*–16:OH, 0.33; and 10*E*,12*E*–16:Ac, 10. Trial conducted June 30, 1998. Total number of moths trapped was 281. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for additive effect, $F = 26.8$, $df = 2, 18$, $P < 0.001$.

pheromone for the saturniid moths *Hemileuca eglanterina* (McElfresh and Millar, 1999a) and *H. nuttalli* (Strecker) (McElfresh and Millar, 1999b). Ongoing studies further indicate that several other species may be using this compound as a pheromone component as well. The 10*E*,12*E*–16 : Ac isomer also has been identified in several other species of hemileucine moths, such as *H. eglanterina* and *H. nuttalli*, but its importance as part of the blend has been demonstrated only for *H. nuttalli*. 10*E*,12*Z*–16 : OH has been identified from both *H. eglanterina* and *H. nuttalli* extracts, but it appears to be an important component of the attractive pheromone blend only for the former species.

As in previously reported studies (McElfresh and Millar, 1999a,b), the use of the MTAD derivatives of the conjugated diene pheromone components proved extraordinarily useful, both in terms of the crucial information that resulted (confirmation of diene positions and molecular weights) and because of the ease and sensitivity of the test. The derivatives were formed by simply mixing the crude pheromone extract with a dilute solution of MTAD, followed by concentration and injection on the GC-MS. Furthermore, because fragmentation of the derivatives occurred primarily by one pathway (cleavage of the alkyl chains from either side of the ring structure), the resulting intense fragment peaks provided diagnostic information even at subnanogram levels. However, for best results, it was crucial to increase the injector temperature to 300°C to transfer the relatively nonvolatile derivatives to the head of the column efficiently.

The amount of 10*E*,12*E*–16 : Ac in female extracts agrees well with that of the synthetic blend which attracted best in the field trials. However, the same was not true for 10*E*,12*Z*–16 : OH, in which the amount found in female extracts appeared to be much higher than the optimal lure ratio. This has also been

observed in both *H. eglanterina* and *H. nuttalli* (McElfresh and Millar, 1999a,b), suggesting that 10*E*,12*Z*–16 : OH is a precursor for 10*E*,12*Z*–16 : Ac and that variation in amounts in gland extracts reflects both materials for release and for processing into other components.

Preliminary data suggests that the congener *Coloradia doris* Barnes may be using a similar pheromone blend (McElfresh and Millar, unpublished data). Responses of male *C. doris* antennae to synthetic standards and *C. velda* female extracts were indistinguishable from those of *C. velda* males in GC-EAD trials. Furthermore, a number of *C. doris* males were attracted to lures containing the three-component blend used to attract *C. velda*. However, this is not surprising considering that the two species are so closely related that they were at one time considered one species.

In summary, the data indicate that the sex pheromone of *C. velda* consists of a combination of 10*E*,12*Z*–16 : Ac, 10*E*,12*E*–16 : Ac, and 10*E*,12*Z*–16 : OH. Deletion of either the 10*E*,12*E*–16 : Ac or 10*E*,12*Z*-16 : OH reduced attraction significantly but not entirely. The fourth antennal stimulatory compound in extracts, tentatively identified as 10*E*,12*Z*-16 : Ald, does not appear to be essential for attraction and actually antagonized male responses at doses of 1 μg or higher. This component, or other minor components in the extracts, may have a role as an antagonist inhibiting the attraction of sympatric congeners.

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ESSENTIAL OILS AS ALLELOCHEMICALS AND THEIR POTENTIAL USE AS BIOHERBICIDES

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Abstract—Essential oils were extracted from 32 aromatic plants and evaluated for allelopathic properties. The major components of the oils were identified by gas chromatography and GC-MS. Extracts from *Origanum syriacum*, *Micromeria fruticosa*, and *Cymbopogon citratus* were selected for further study. The germination of several species, including wheat, was strongly inhibited by essential oils when applied at 20–80 ppm. Essential oils mixed with the top 0.5 cm of soil inhibited germination of wheat and *Amaranthus* seeds. This effect depended on the type of soil. The possible use of essential oils as herbicides is discussed.

Key Words—Allelochemicals, carvacrol, citral, essential oils, geranial, herbicides, inhibition of germination, pulegone.

INTRODUCTION

Potential damage to human health and to the environment from herbicides is regarded as a real problem today. It has resulted in an increased interest in alternative strategies leading to the development of biodegradable compounds (Wyse, 1994; Buhler, 1996; Jordan, 1996).

Inhibition of growth of plants by other plants in their vicinity has been known for a long time. The chemical interaction between plants, which can cause enhancement or inhibition of growth, has been named allelopathy (Molisch, 1937; Rice, 1984). Evidence for allelopathic interactions in nature by plants containing volatile

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allelochemicals has been described frequently (Sigmund, 1924; Went, 1942; Bonner, 1950; Asplund, 1968; Chou, 1986, 1989; Muller, 1986; Reynolds, 1987).

Most of the germination and growth inhibitors produced by perennial angiosperms identified by Rice (1984) were phenolic compounds or derivatives of cinnamic acid. Other authors also found coumarins, flavonoids, alkaloids, cyanoglycosides, proteins, and amino acids among the inhibitory compounds (Friedman and Walker, 1983; Putnam, 1985; Waller, 1989). To this list the terpenoids must be added, including the volatile terpenes that are the main components of essential oils (Fischer, 1986; Muller, 1986; Elakovich, 1988). Muller et al. (1964) and Muller (1965) reported that in the vicinity of aromatic shrubs, such as whiteleaf sage (*Salvia leucophylla* Greene) or California sagebrush (*Artemisia californica* Less.), there were no annual plants within a diameter of 90 cm, and presence of annuals was very limited within 2–6 m. Muller et al. (1964) and Friedman et al. (1977) found that volatile foliage compounds were the active ingredients causing the repression of growth in the vicinity of the *Salvia* and *Artemisia* species. This ecological phenomenon provides a competitive advantage to aromatic plants in their natural environments. These reports suggest that allelochemicals could be used for weed control in agriculture. Species of *Amaranthus*, *Sinapis*, and *Euphorbia* are very widespread weed species throughout Israel and coexist with *Origanum syriacum* and *Micromeria fruticosa.* In the immediate vicinity of the two latter species, very few other plants grow or germinate. We report the activity of essential oils as growth and germination inhibitors of these and other species and their possible use as herbicides.

METHODS AND MATERIALS

Essentials Oils

Essential oils were hydrodistilled for 1.5 hr from 250 g of shoots of fresh aromatic plants harvested in July from experimental plots of the Newe Ya'ar Research Center, Israel, using a modified Clevenger apparatus (Ravid et al., 1997). Oils from common caraway (*Carum carvi* L.) and coriander (*Coriandrum sativum* L.) were extracted from 100 g dry seed. The composition of the essential oils in the distillates was identified using gas chromatography and GC-MS analysis described previously by Ravid et al. (1997). After preliminary screening of 32 aromatic plants that had relatively high activity, three species, *Origanum syriacum*, *Cymbopogon citratus*, and *Micromeria fruticosa*, were chosen for more detailed studies.

Bioassay of Inhibition Induced by Essential Oils

Seed Germination in Petri Dish. Seeds of wheat (*Triticum aestivum* L.), black mustard [*Brassica nigra* (L.) Koch], palmer amarant (*Amaranthus palmeri* S. Watson), and various aromatic or weed species were germinated in Petri

dishes (6 cm diam.) on three layers of filter paper (Whatman No. 3) wetted with 4 ml of distilled water. Petri dishes containing 20 seeds were incubated in the dark at 27°C. To test the inhibitory effect of an essential oil, a known amount of oil was loaded (using a calibrated glass microcapillary) on a piece of filter paper attached (by double-sided adhesive tape) to the inner side of the cover of the Petri dish. Up to 3 μl of essential oil and blank controls were tested with this procedure. One microliter of essential oil per Petri dish is equivalent to 40 nl/ml. Each amount was tested in five replicates. After incubation, the numbers of germinated seeds were counted and the length of each radicle was measured. The amount of essential oil required to cause 50% inhibition of germination was determined by interpolation from the curve obtained by plotting the means of the replicates against the amount of essential oil applied.

Seedling Growth in Petri Dish. A layer of wheat seeds was germinated, between layers of wet filter paper (Whatman No. 3), and incubated at 27°C for 24 hr. After incubation, 10 seedlings with radicles of 1–2 mm were transferred to each Petri dish, as described above, and it was loaded with 0–1.25 μl of essential oil. After 48 hr of incubation at 27°C, length of radicles and coleoptiles was measured and compared with those of untreated seedlings.

Seedling Emergence in Soil. Plastic pots of 250 ml (8.5 cm diameter, 7.3 cm height) were filled with 230 g/pot of clay soil (from Newe Ya'ar, Israel), 200 g/pot of loam soil (from Rehovot, Israel), or 200 g/pot of loess (from Ein Haslosha, Israel). In each pot 20 seeds of wheat or black mustard, or 45 seeds of *Amaranthus palmeri* were sown at a depth of 1 mm. The first irrigation water was provided from below. Five milliliters of the essential oil suspended in water with 0.5% v/v of a surfactant (Shatah 90, alkyl-phenolethylene oxide, Machteshim, Beer Sheva) was sprayed on the soil surface; this was immediately followed by gently spraying with water. Pots were irrigated daily by gently spraying with water until water drained from the bottom of the pot. Tests of percent emergence were performed in a greenhouse in four replicates.

Statistical Analysis

Replicated experiments were conducted in a completely randomized design. The significance ($P \leq 0.05$) of differences between treatments was determined by Duncan's multiple range test, or using the least significant difference (LSD).

RESULTS AND DISCUSSION

Essential Oils and Germination Inhibition. The response of wheat seeds to an essential oil extracted from *Cymbopogon citratus* is shown in Figure 1. Figure 1D shows a control seed not exposed to oil. Only seeds with radicle emergence as in Figure 1C were considered to have germinated. Seeds with a

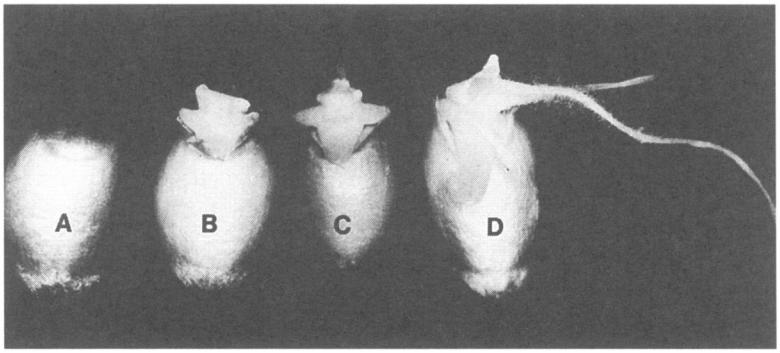

FIG. 1. Response of wheat seed to 40 nl/ml essential oil. Photograph taken 48 hr after sowing. A–C show different responses to the essential oil: (A) seed did not germinate, (B) incomplete germination with lack of radicle emergence, (C) seed did germinate, (D) a control seed, not exposed to oil.

protrusion of nondifferentiated cells without radical emergence were considered not germinated (Figure 1B). The radicle of germinated seeds exposed to essential oil (Figure 1C) was shorter than that of control seeds (Figure 1D).

The effect of essential oils extracted from aromatic plants on germination of wheat seeds is shown in Figure 2 and Table 1. For most of the extracted oils, 50% inhibition of germination was observed between 28 and 84 nl/ml of essential

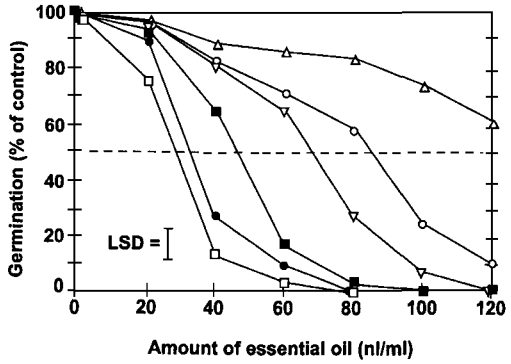

FIG. 2. Effect of essential oils on germination of wheat seed. Wheat seed was incubated for 48 hr in the presence of different amounts of essential oil per Petri dish. Essential oils were extracted from: □, *Micromeria fruticosa*; ●, *Cymbopogon citratus*, ■, *Ocimum citriodorum*; ▽, *Origanum majorana*; ○, *Pelargonium graveolens*; and △, *Artemisia absinthium*.

TABLE 1. INHIBITION OF GERMINATION OF WHEAT SEED BY ESSENTIAL OIL EXTRACTED FROM AROMATIC PLANTS

Species	I_{50} (nl/ml)[a]
Micromeria fruticosa	28
Artemisia judaica (Israeli var.)	28
Mentha piperita	28
Cymbopogon citratus	32
Artemisia judaica (Sinai Var.)	32
Mentha longifolia	36
Melissa officinalis	40
Salvia officinalis (Dalmatian var.)	40
Eucalyptus citriodora	40
Ocimum citriodorum	40
Rosmarinus officinalis	40
Artemisia arborescens	48
Carum carvi	48
Lavendula officinalis	52
Thymus citriodorus	52
Ocimum basilicum	52
Hyssopus officinalis	56
Coriandrum sativum	56
Coridothymus capitatus	60
Origanum syriacum (thymol chemotype)	60
Origanum vulgare (thymol chemotype)	64
Origanum majorana	68
Lippia citriodora	68
Origanum syriacum (Carvacrol chemotype)	68
Thymus vulgaris	76
Myrtus communis	76
Laurus nobilis	84
Pelargonium graveolens	84
Ocimum basilicum (Methyl chavicol type)	>100
Artemisia dracunculus (Russian var.)	>100
Artemisia dracunculus (French var.)	>100
Artemisia absinthium	>100
LSD (0.05)	12

[a]Values presented are amount of essential oil per unit volume required to cause 50% inhibition of germination, obtained by interpolation.

oil. The essential oils from four plants, *Ocimum basilicum*, *A. dracunculus* (two varieties), and *A. absinthium*, were ineffective in inhibiting germination at the concentration evaluated.

The inhibition of germination of seeds from aromatic plants by essential oils extracted from the same and some different aromatic plants was tested (Table 2). The distillate containing essential oils from four species inhibited germination

TABLE 2. INHIBITION OF GERMINATION OF AROMATIC PLANTS BY ESSENTIAL OIL EXTRACTED FROM AROMATIC PLANTS

Species	I_{50} (nl/ml)[a]					
	Ocimum basilicum	Salvia sclarea	Carum carvi	Melissa officinalis	Coriandrum sativum	LSD (0.05)
Ocimum basilicum	100[b]	100	100	56	68	12
Salvia sclarea	56	88	36	68	28	8
Carum carvi	32	52	52	32	52	12
Melissa officinalis	72	68	12	20	12	8
Triticum aestivum	108	92	48	76	44	8
Sinapis nigra	100	108	84	36	104	8
Lycopersicum esculentum	44	100	32	52	76	8

[a]Values presented are amount of essential oil per unit volume required to cause 50% inhibition of germination, obtained by interpolation. Plants in column heads are the sources of the essential oils.
[b]Underlined values show inhibition of seeds by the essential oil of the same species.

of seeds of the plant from which they were extracted as well as the germination of other species. Moreover, seed of some species was much less sensitive to certain essential oils compared with other essential oils; compare, for example, the effect of *Ocimum* oil on *Sinapis* and *Carum.*

The curve of percent inhibition versus concentration of essential oil from lemon grass (*Cymbopogon citratus*), whose major component is citral, is sigmoidal. In contrast, the curve from oregano oil (*Origanum vulgare* L.), whose major component is carvacrol, is linear in the same concentration range (Figure 3). This suggests that the mechanism of action of these essential oils may be

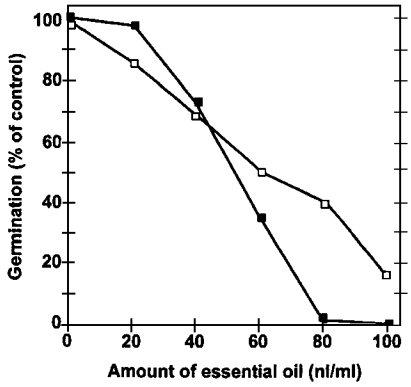

FIG. 3. Inhibition of germination of wheat seeds by essential oils from *Cymbopogon* (■) and *Origanum* (□).

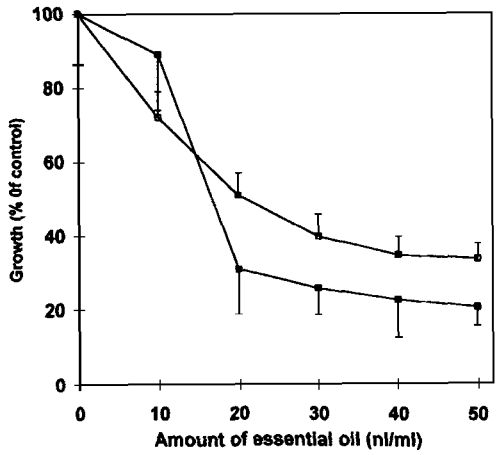

FIG. 4. Inhibition of wheat seedling radicle and coleoptile growth by essential oil extracted from lemon grass: ■, radicle; □, coleoptile.

different. Similar results are obtained when pure compounds are used (data not shown).

Since essential oils inhibit both germination and growth (Figure 1), the effect of essential oil of lemon grass on wheat seedlings was studied. Fifty percent inhibition of growth of radicles occurred at 16 nl/ml of lemon grass oil as compared with 32 nl/ml required for 50% inhibition of germination (Table 1, Figure 4). Radicle growth was more sensitive to essential oil inhibition than coleoptile growth; the difference was small (Figure 4) but reproducible in all the assays.

Table 3 shows the composition of the essential oils of three species with good inhibitory activity. The major components of these oils represent different types of monoterpenes: phenolic (carvacrol) in *O. syriacum*; ketonic (pulegone) in *M. fruticosa*; aldehydic (geranial and neral) in *C. citratus*. The data in Table 4 show that the germination of six species differed markedly in their response to the three extracts containing the essential oils.

Most investigations on allelopathy have characterized the phenomenon but did not consider applications in agriculture. In the present study, we have examined the first steps towards a possible practical application. We therefore tested whether compounds that affect germination in the Petri dish show a similar effect in soil.

The data show that essential oils not only inhibit germination and growth of plants in Petri dishes but also in soil (Tables 5–7). The most active essential oil was the aldehyde from lemon grass, and it was therefore chosen for further

TABLE 3. MAIN COMPONENTS OF THREE ESSENTIAL OILS OF THREE AROMATIC PLANTS

Component	% of total essential oil		
	Origanum syriacum	*Micromeria fruticosa*	*Cymbopogon citratus*
Carvacrol	60.1		
β-Caryophyllene	1.7	5.1	2.4
p-Cymene	19.7		
Geranial[a]			42.6
Neral[a]			32.1
Geraniol			3.3
Isomenthol		11.5	
Limonene		2.9	
Linalol			2.2
Menthol		1.3	
Myrcene	2.8		10.4
α-Pinene	3.6	1.4	
Pulegone		59.7	
α-Terpinene	2.5		
γ-Terpinene	13.0		

[a]These two compounds, which are *cis–trans* isomers, constitute citral.

study. Inhibition of wheat, *Amaranthus palmeri*, and *Brassica nigra* seed by the essential oil was generally more effective in loam or loess than in clayey soil (Tables 5 and 7). The inhibition of germination decreased with increase of sowing depth.

TABLE 4. INHIBITION OF GERMINATION OF SEED OF FOUR WEEDS AND TWO CROP PLANTS BY ESSENTIAL OILS OF THREE SPECIES

Species tested	I_{50} (nl/ml)[a]			
	Origanum syriacum	*Micromeria fruticosa*	*Cymbopogon citratus*	LSD (0.05)
Amaranthus blitoides	36	40	20	8
Amaranthus palmeri	20	28	8	12
Euphorbia hirta	100	116	92	20
Sinapis nigra	96	72	8	8
Trifolium campestre	104	56	56	8
Lycopersicum esculentum	8	16	24	12

[a]Values presented are amount of essential oil per unit volume required to cause 50% inhibition of germination, obtained by interpolation. Plants in column heads are the sources of the essential oils.

TABLE 5. EFFECT OF 1% LEMON GRASS ESSENTIAL OIL SUSPENSION AND SOWING DEPTH ON EMERGENCE OF WHEAT AND MUSTARD IN TWO SOILS

Depth of sowing (mm)	Emergence (% of control)			
	Wheat		Mustard	
	Clay soil	Loam	Clay soil	Loam
1	86 a[a]	32 b	13 B	0 C
5	86 a	82 a	15 B	43 AB
10	83 a	84 a	90 A	87 A

[a]Different letters for wheat (lower case), and for mustard (upper case) beside values indicate values significantly different at $P \leq 0.05$.

The results of this study show that there are differences in the sensitivity of the various species (Tables 2 and 4). There are also differences in potency of the essential oils (Tables 1, Figure 2). Seeds that germinated in presence of essential oils usually did not develop normally. The growth of their radicles and coleoptiles was inhibited compared with the control (Figure 1). Fifty percent inhibition of growth occurred at a lower oil concentration than for germination, and radicle growth was somewhat more sensitive than coleoptile growth (Table 1, Figure 4). The fact that, in a given species, germination is partly inhibited by

TABLE 6. EFFECT OF THREE AROMATIC PLANT ESSENTIAL OIL SUSPENSIONS ON EMERGENCE OF WHEAT AND PALMER AMARANTH SEEDS IN CLAYEY SOIL[a]

Species	Concentration (%)	Emergence (% of control)[b]		
		Origanum syriacum	*Micromeria fruticosa*	*Cymbopogon citratus*
Wheat	0.0	100 A	100 A	100 A
	0.5	90 A	95 A	71 B
	1.0	85 A	85 AB	34 C
	1.5	37 B	75 B	33 C
	2.0	10 C	67 B	0 D
Amaranth	0.0	100 a	100 a	100 a
	0.5	99 a	97 a	36 b
	1.0	98 a	69 b	22 bc
	1.5	56 b	61 b	11 c
	2.0	0 c	56 b	8 c

[a]Data shown for emergence 8 days after sowing.
[b]Within columns, different letters for wheat (upper case) and for palmer amaranth (lower case) beside values indicate values significantly different at $P \leq 0.05$.

TABLE 7. EFFECT OF LEMON GRASS ESSENTIAL OIL SUSPENSION ON EMERGENCE OF WHEAT AND PALMER AMARANTH SEED IN THREE SOILS[a]

Species	Concentration (%)	Emergence (% of control)		
		Clay soil	Loam	Loess
Wheat	0.0	100 A[1]	100 A	100 A
	0.5	62 B	31 B	16 B
	1.0	9 C	10 BC	11 B
	1.5	0 C	0 C	0 C
	2.0	0 C	0 C	0 C
Amaranth	0.0	100 a	100 a	100 a
	0.5	55 b	4 b	12 b
	1.0	12 c	0 b	12 b
	1.5	10 c	0 b	0 c
	2.0	0 c	0 b	0 c

[a]Seeds were sown at 1 mm depth. Values were determined 8 days after sowing.
[b]Within columns, different letters for wheat (upper case) and for palmer amaranth (lower case) beside values indicate values significantly different at $P \leq 0.05$.

a given essential oil does not imply that the germinated seeds will develop into a normal seedling. It seems probable that growth inhibition prevents the normal penetration of the root into the soil. After leaf fall in summer, in dense populations of *Origanum syriacum* and *Micromeria fruticosa*, almost no other plants develop near the aromatic plants. In pot experiments, in which leaves of these species were incorporated into the top layer of the soil, there was marked inhibition of growth and germination (data not shown). The observations support the view that allelopathic interactions do indeed occur. These data suggest that essential oils could be used to inhibit emergence of weeds. Further studies, especially on formulation of essential oils for application, are still required to apply this technique to agriculture.

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SYNERGISTIC SEX PHEROMONE COMPONENTS OF WHITE-SPOTTED TUSSOCK MOTH, *Orgyia thyellina*

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Abstract—In 1996, the exotic white-spotted tussock moth (WSTM), *Orgyia thyellina* (Lepidoptera: Lymantriidae), was discovered in Auckland, New Zealand. Because establishment of WSTM would threaten New Zealand's orchard industry and international trade, eradication of WSTM with microbial insecticide was initiated. To monitor and complement eradication of WSTM by capture of male moths in pheromone-baited traps, pheromone components of female WSTM needed to be identified. Coupled gas chromatographic–electroantennographic detection analysis of pheromone gland extract revealed several compounds that elicited responses from male moth antennae. Mass spectra of the two most EAD-active compounds suggested, and comparative GC-MS of authentic standards confirmed, that they were (*Z*)-6-heneicosen-11-one (*Z*6–11-one) and (*Z*)-6-heneicosen-9-one, the latter termed here "thyellinone." In field experiments in Japan, *Z*6–11-one plus thyellinone at a 100:5 ratio attracted WSTM males, whereas either ketone alone failed to attract a single male moth. Addition of further candidate pheromone components did not enhance attractiveness of the binary blend. Through the 1997–1998 summer, 45,000 commercial trap lures baited with 2000 μg of *Z*6–11-one and 100 μg of thyellinone were deployed in Auckland towards eradication of the residual WSTM population.

Key Words—Lepidoptera, Lymantriidae, white-spotted tussock moth, *Orgyia thyellina*, (*Z*)-6-heneicosen-11-one, (*Z*)-6-heneicosen-9-one, (*Z*)-6,(*E*)-8-

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[4]Dedicated to my dear mother in honor of her 75th birthday.

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heneicosadien-11-one, sex pheromone, synergism, quarantine insect, international trade, eradication, *Bacillus thuringiensis*, microbial insecticide.

INTRODUCTION

In April 1996, white-spotted tussock moths (WSTM), *Orgyia thyellina* (Lepidoptera: Lymantriidae), were discovered in suburbs of Auckland City, New Zealand. WSTM is native to eastern Asia, including Japan (Inoue, 1956), Korea (Kim et al., 1982), the Russian Far East (Kozhanchikov, 1950), eastern China (Zhao, 1982), and Taiwan (Inoue, 1956, but see Kishida, 1992). Caterpillars feed on a variety of host plants in the rose (Rosaceae), willow (Salicaceae), birch (Betulaceae), legume (Fabaceae), oak (Fagaceae), mulberry (Moraceae), ebony (Ebenacae), elm (Ulmacae), and maple (Aceraceae) families (Kozhanchikov, 1950; Kim et al., 1982). In Japan and northern populations, there are two or three generations per year in which female WSTM show wing dimorphism. They are fully winged and flight capable in the spring or summer generation(s), but wingless and mainly sedentary in the fall generation (Inoue, 1956; Kimura and Masaki, 1977). With this considerable potential to disperse and the wide range of host plants, WSTM's spread and establishment throughout New Zealand was very likely. WSTM could become New Zealand's most serious defoliator of pip, stone, and berry fruits and may also become a serious pest of urban amenity plantings. Because female WSTM oviposit also on wooden crates, pallets, logs, containers, and other export material, and because egg masses are resilient, establishment of WSTM would likely affect New Zealand's international trade. A direct risk from WSTM to human health stems from the urticating hairs produced by caterpillars. Scientists working with the moth developed respiratory problems and rashes. Once sensitized, people exposed to the hairs may develop symptoms at increasingly low exposure. Furthermore, establishment of WSTM in urban environments would lead to increased and widespread use of chemical insecticides by both local authorities and home owners (Hosking, 1997).

In October 1996, New Zealand's Ministry of Forestry launched "Operation Ever Green" aimed at eradicating WSTM. This eradication program used a combination of aerial and ground spraying of the microbial insecticide *Bacillus thuringiensis* var. *kurstaki* (*Btk*). In the first phase of the operation (October–December 1996), 40 km^2 of Auckland were treated nine times from the air with a DC-6 aircraft and ~300 properties were sprayed from the ground using mist blowers. In the second phase (January–April 1997), 3 km^2 were treated 14 times with a helicopter, and ~200 properties were sprayed from the ground (Hosking, 1997).

Capture of male moths in traps baited with WSTM females were employed to assess progress towards eradication of WSTM. However, supply of female

moths for trapping of males was interrupted when a virus infection caused collapse of the WSTM colony reared in a quarantine facility. If sex pheromone components of female WSTM were known, synthetic pheromone baits—instead of female moths—could be emloyed as trap baits. Large numbers of pheromone-baited traps would provide a continuous measure of the residual WSTM population and would complement other eradication efforts, such as *Btk* sprays. We report identification and field testing of WSTM sex pheromone components.

METHODS AND MATERIALS

Insect Culture and Pheromone Extraction

WSTM caterpillars were reared on apple foliage (photoperiod: 14L : 10D; temperature: 24°C; relative humidity: 55–60%) at the Forestry Research Institute Rotorua, Auckland, New Zealand. Abdominal tips with pheromone glands of calling, 1- to 2-day-old virgin female moths were removed and placed in redistilled hexane. After 30–60 min of extraction, the supernatant was withdrawn, syringed into ampoules, and shipped (together with male pupae) to Simon Fraser University.

Laboratory Analyses, Instruments, and General Procedures

Aliquots of 1 female equivalent (FE) of pheromone gland extract were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), using a Hewlett Packard (HP) 5890A gas chromatograph fitted with an on-column injector and equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with either DB-210, DB-5, or DB-23 (J&W Scientific, Folsom, CA 95630). GC–mass spectrometry (MS) of synthetic or antennally active WSTM-produced compounds in full scan electron impact mode employed a Varian Saturn II Ion Trap GC-MS fitted with the DB-210 column referred to above. Synthetic (*Z*)-6,(*E*)-8-heneicosadien-11-one (Gries et al., 1997) was purified by high-performance liquid chromatography (HPLC) with a Waters LC 626 high-performance liquid chromatograph equipped with a Waters 486 variable wavelength UV visible detector set to 220 nm, an HP integrator, and a Nova Pak C_{18} (3.9 × 300 mm) column with 1 ml/min of acetonitrile flow. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Bruker AMX-400 spectrometer at 400.13 MHz for ^{1}H NMR spectra. ^{1}H chemical shifts are reported as parts per million (ppm; δ) relative to TMS (0.00 ppm). Elemental analyses were performed using a Carlo Erba model 1106 elemental analyzer. Chemicals obtained from commercial sources were used without further purification unless otherwise indicated. All moisture- and air-sensitive reactions were conducted under argon. Column chromatogra-

phy refers to flash chromatography on silica gel 60 (230–400 mesh, E. Merck, Darmstadt, Germany) (Still et al., 1978).

Syntheses

6-Heneicosyn-9-ol (4) (Figure 1, Scheme 1). 1-Tetradecane (**2**) obtained from myristyl bromide (**1**) with lithium diisopropylamide (LDA) at 0°C was converted with *m*-Cl-peroxybenzoic acid to 1,2-epoxy-tetradecene (**3**) (87% yield). Epoxide **3** (4.50 g, 21.2 mmol) was added under argon to a prepared reaction mixture of heptyne (5.25 ml, 40 mmol), BuLi (40 mmol), and hexamethylphospho rous triamide (HMPA) (30 ml) in 200 ml of dry tetrahydrofuran (THF) at −30°C.

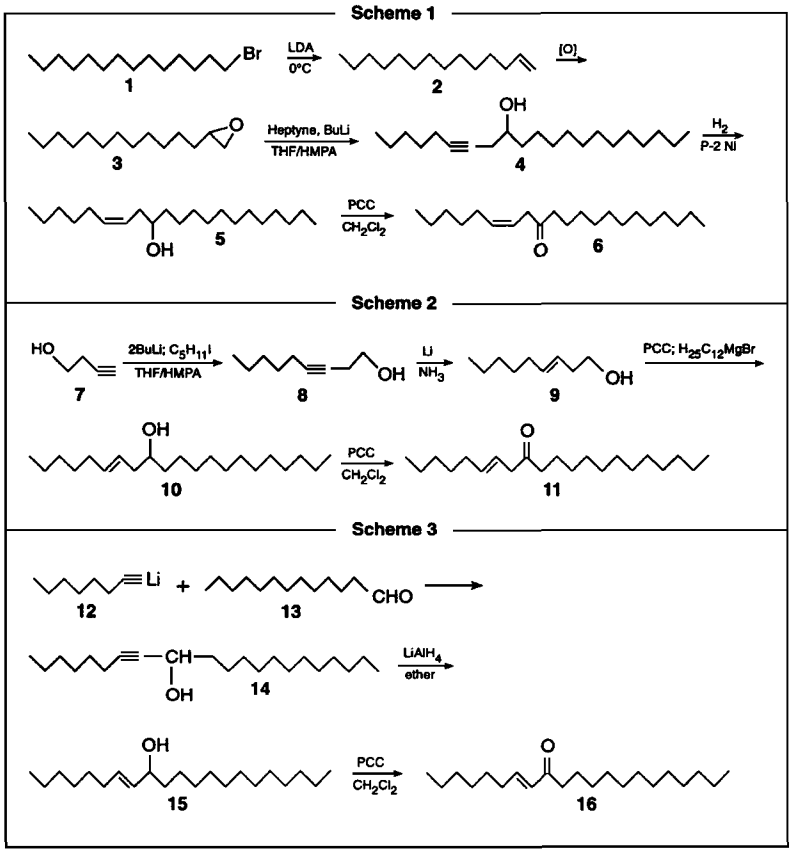

FIG. 1. Schemes for the syntheses of (*Z*)-6-heneicosen-9-one (Scheme 1), (*E*)-6-heneicosen-9-one (Scheme 2), and (*E*)-7-heneicosen-9-one (Scheme 3).

The mixture was gradually warmed to 50°C and kept 4 hr under argon with stirring. It was then cooled to room temperature and quenched with NH_4Cl. It was extracted (3 × 100 ml) with hexanes–ether (1 : 1). The organic layer was washed with saturated NaCl, dried, and purified by column chromatography [hexanes–ether (98 : 2)], and gave **4** (6.35 g, 92% yield; mp: 31°C). Anal. calcd. for $C_{21}H_{40}O$: C, 81.73; H, 13.07; found: C, 81.72; H, 13.02; ^1H NMR: 3.68 (m, 1H); 2.40, 2.26 (ab quartet, 2H, split further into double triplets due to long range coupling); 2.17 (tt, 2H); 1.50 (m, 2H); 1.22–1.40 (m, 26H), 0.87 (t, 3H), 0.89 (t, 3H).

(Z)-*6-Heneicosen-9-ol (5) (Figure 1, Scheme 1).* Alcohol **4** (6.30 g, 20 mmol) was hydrogenated with P-2 Ni, and the reaction was monitored by GC. The resulting olefinic alcohol (**5**) was column chromatographed [hexanes–ether (95 : 5)], yielding 5.80 g of **5** with 93.5% chemical purity. Anal. calcd. for $C_{21}H_{42}O$: C, 81.20; H, 13.63; found: C, 80.89; H, 13.97. ^1H NMR: 5.56 (m, 1H); 5.40 (m, 1H); 3.61 (m, 1H); 2.21 (dd, 2H); 2.05 (dt, 2H); 1.23–1.48 (m, 28H), 0.88 (m, 6H).

(Z)-*6-Heneicosen-9-one (6) (Figure 1, Scheme 1).* Alcohol **5** (6.0 g, 19.3 mmol) was stirred 90 min in 20 ml of CH_2Cl_2 with pyridinium chlorochromate (PCC) (6.25 g, 28.5 mmol). Hexane (30 ml) was added and the mixture column chromatographed [hexanes–ether (99 : 1)]. Ketone-containing fractions, monitored by GC, were combined yielding 5.1 g of the ketone **6** (85.5% yield). Anal. calcd. for $C_{21}H_{40}O$: C, 81.73; H, 13.07; found: C, 81.69; H, 13.22. ^1H NMR: 5.56 (m, 2H); 3.15 (d, 2H); 2.42 (t, 2H); 2.02 (m, 2H); 1.55 (m, 4H); 1.20–1.42 (m, 22H); 0.88 (m, 6H).

(E)-*6-Heneicosen-9-ol (10) (Figure 1, Scheme 2).* Butyl lithium-induced coupling of 3-butyn-1-ol (**7**) with iodopentane in the presence of HMPA, and subsequent reduction of the resulting 3-nonyn-1-ol (**8**) with lithium in liquid ammonia afforded (*E*)-3-nonen-1-ol (**9**) (Katritzky et al., 1996) at 36% yield (based on **7**). It was purified (98%) on a silica column containing 20% silver with hexane–ether–benzene (3 : 1 : 2) as eluents. To alcohol **9** (1.2 g, 8.45 mmol) in CH_2Cl_2 (10 ml) at room temperature, PCC (12.7 mmol) was added. After 1 hr of stirring, the reaction mixture was quenched with hexane (30 ml) and flash chromatographed with hexane–ether (10 : 1). Aldehyde-containing fractions, as monitored by GC, were combined, concentrated to 10 ml, placed into a dropping funnel, and added to a Grignard reagent freshly prepared from dodecyl bromide (10 mmol) and magnesium (12 mmol) in ether. After 15 min of stirring, the reaction mixture was gently quenched with aqueous ammonium chloride. Extraction (3 × 100 ml of ether), separation, and washing of organic layers with saturated NaCl, drying, and column chromatography [hexane–ether (98 : 2)] yielded alcohol **10** (1.50 g, 57% yield) as a solid compound (mp 32–33°C); anal. calcd. for $C_{21}H_{42}O$: C, 81.20; H, 13.63; found: C, 80.70; H, 13.85; ^1H NMR: 5.54 (m, 1H); 5.40 (m, 1H); 3.57 (m, 1H); 2.23 (m, 1H); 1.98–2.09 (m, 3H); 1.21–1.46 (m, 28H); 0.87 (two t, 6H).

(E)-*6-Heneicosen-9-one (11) (Figure 1, Scheme 2).* Ketone **11** was obtained

(85% yield) from alcohol **10** following the procedure applied for conversion of compound **5** to **6.** Anal. calcd. for $C_{21}H_{40}O$: C, 81.73; H, 13.07: found; C, 81.39; H, 13.03; ^{1}H NMR: 5.51 (m, 2H); 3.08 (d, 2H); 2.41 (t, 2H); 2.02 (m, 2H); 1.5–1.6 (m, 4H); 1.18–1.38 (m, 22H); 0.88 (m, 6H).

(E)-*7-Heneicosen-9-one (16) (Figure 1, Scheme 3).* GC analysis of synthetic ketone **6** resulted in the formation of rearrangement products [4–10% of **6** depending on the GC injector (split/splitless or on-column)]. Heat exposure of ketone **6** (5 mg) (180°C for 5 min under argon) and subsequent two-dimensional ^{1}H NMR spectroscopy suggested, and synthesis confirmed, that the major rearrangement product was (*E*)-7-heneicosen-9-one (**16**). For synthesis of **16** (Figure 1, Scheme 3), lithium octynide (**12**) was coupled with tridecanal (**13**), affording 7-heneicosyn-9-ol (**14**), which was reduced overnight with $LiAlH_4$ in ether to (*E*)-7-heneicosen-9-ol (**15**) and oxidized with PCC to give **16** (62% overall yield). ^{1}H NMR: 6.82 (two t, 1H); 6.08 (td, 1H); 2.52 (t, 2H); 2.20 (q, 2H); 1.59 (m, 2H); 1.45 (m, 2H); 1.21–1.35 (m, 22H), 0.86 (m, 6H).

The following compounds were produced to confirm that their GC retention times differed from that of Z6–9-one (Figure 1, Scheme 1). Because these compounds are not synthetic intermediates for pheromones and do not have pheromonal activity, their syntheses are reported without spectroscopic data or schemes, as follows:

(Z)-*4-Heneicosen-9-one.* 5-Hexyn-1-ol was added to 2.5 equivalents of butyl lithium in THF. Addition of bromopropane in HMPA resulted in the formation of 5-nonyn-1-ol. Hydrogenation over P-2 Ni followed by oxidation with PCC gave (*Z*)-5-nonenal, which was added to a solution of dodecyl magnesium bromide, resulting in (*Z*)-4-heneicosen-9-ol. Oxidation with PCC yielded (*Z*)-4-heneicosen-9-one.

(Z)-*5-Heneicosen-9-one.* 4-Pentyn-1-ol was added to 2.5 equivalents of butyl lithium in THF, followed by bromobutane in HMPA. Oxidation of the resulting 4-nonynol with PCC provided the aldehyde, which was added to dodecylmagnesium bromide, resulting in 5-heneicosyn-9-ol. Oxidation with PCC and hydrogenation over P-2 Ni yielded (*Z*)-5-heneicosen-9-one.

(E)-*5-Heneicosen-9-one.* 4-Nonyn-1-ol was treated with lithium in liquid ammonia to form (*E*)-4-nonen-1-ol, which was oxidized with PCC to (*E*)-4-nonenal. Reaction with dodecyl magnesium bromide and oxidation as above provided (*E*)-5-heneicosen-9-one.

(*Z*)-6,(*E*)-8-Heneicosadien-11-one (*Z*6,*E*8–11-one) and (*Z*)-6-heneicosen-11-one (Z6–11-one) were available from previous research (Gries et al., 1997). (*Z*)-6,(*Z*)-9-Heneicosadiene (*Z*6,*Z*9–21-Hy) was generously provided by the National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan.

All synthetic chemicals used for GC-EAD, wind-tunnel, and field bioassays were >95% chemically pure.

Wind-Tunnel, Field-Cage, and Field Experiments

Wind-Tunnel Bioassays. In the bioassays we employed a wind tunnel and protocol as developed by Miller and Roelofs (1978). The airspeed was 30 cm/sec and the temperature was 20°C (±2°C). Both bioassay experiments tested gray sleeve stopper (The West Company, Lionville, Pennsylvania 19341) lures impregnated with test chemicals in HPLC grade hexane. Six criteria (Table 1) were used to assess the attractiveness of lures. The first bioassay experiment tested Z6–11-one alone and in combination with either Z6–9-one or E6–9-one. The second bioassay experiment tested Z6–11-one alone and in combination with either Z6–9-one, Z6,E8–11-one, or both.

Field-Cage Bioassays. Field-cage bioassays were conducted in Aukland, New Zealand, employing a cage (10 × 4 × 2.4 m high) with a steel tubing frame and heavy mesh cover. Experimental replicates (*N*) were conducted from 10:00 AM to 1:00 PM on January 13–15 (*N:* 1–3) and January 27–29 (*N:* 4–6). Each replicate employed 10 green delta traps (IPM Technologies, Inc., Portland, Oregon 97217) suspended 2 m above ground at 2-m spacings, with rerandomized positions between replicates. Five traps were baited with a commercial formula-

TABLE 1. WIND-TUNNEL BIOASSAYS WITH MALES OF WHITE-SPOTTED TUSSOCK MOTH, *Orgyia thyellina*, RESPONDING TO GRAY SLEEVE STOPPERS IMPREGNATED WITH CANDIDATE PHEROMONE COMPONENTS

Exp.[a]	Treatment[b]	Percent males responding to assessment criteria[c]					
		I	O	H	A	L	T
1	Z6–11-one (100)	0	0	0	0	0	0
	Z6–11-one (100) + E6–9-one (1)	0	0	0	0	0	0
	Z6–11-one (100) + E6–9-one (10)	0	0	0	0	0	0
	Z6–11-one (100) + Z6–9-one (1)	100	100	75	75	0	25
	Z6–11-one (100) + Z6–9-one (10)	50	50	50	50	0	0
2	Z6–11-one (100)	17	0	0	0	0	0
	Z6–11-one (100) + Z6,E8-11-one (2)	12	0	0	0	0	0
	Z6–11-one (100) + Z6–9-one (2)	68	39	12	12	12	10
	Z6–11-one (100) + Z6–9-one (2) + Z6,E8–11-one (2)	80	73	54	54	54	46

[a]Bioassay experiments 1 and 2 tested 8 and 41 male moths, respectively, for each of the treatments.
[b]Z6–11-one = (Z)-6-heneicosen-11-one; Z6–9-one = (Z)-6-heneicosen-9-one; Z6,E8–11-one = (Z)-6,(E)-8-heneicosadien-11-one; Z6,Z9–21-Hy = (Z)-6,(Z)-9-heneicosadiene. Numbers in parentheses are micrograms.
[c]I = initiation of flight; O = orientation towards pheromone lure, H = halfway flight towards lure, A = approaching lure, L = landing on platform that carries lure, T = touching and attempting to mate with lure.

tion (IPM Technologies) of Z6–11-one (2000 μg) plus thyellinone (100 μg), and five traps were baited with a virgin female WSTM. Each female was retained in a stainless steel wire mesh cylinder with plywood discs at the top and bottom. Because females lack functional mouthparts, no food or water was provided. The same females were used for replicates 1–3 and 4–6, respectively. Numbers of active males per replicate ranged between 17 and 44.

Field Experiments. Field experiments were conducted in and around Morioka, Japan. Green belts and forest margins stocked with decidious trees and various shrubs and herbs served as experimental sites. Sticky 2-liter Delta milk carton traps (Gray et al., 1984) were suspended from trees 1.5 m above ground in randomized complete blocks. Traps within blocks (= replicates) were spaced at intervals of 15–20 m, whereas replicates (in response to low WSTM populations) were set 0.1–4 km apart from another. Traps were baited with gray sleeve stoppers impregnated with synthetic candidate pheromone components in HPLC grade hexane. The first experiment tested Z6–11-one in binary, ternary, and quaternary combinations with Z6–9-one, Z6,*E*8–11-one, and Z6,Z9–21-Hy. Experiment 2 tested Z6–11-one alone and in combination with Z6–9-one at ratios of 100 : 10 and 100 : 1. Experiments 3–5 tested Z6–11-one plus Z6–9-one alone and in combination with either Z6,Z9–21-Hy (experiment 3) or Z6,*E*8–11-one (experiments 4 and 5) at different ratios. Experiment 6 tested Z6–11-one and Z6–9-one singly and in combination. The final experiment 7 tested increasing doses of Z6–11-one, Z6–9-one and Z6,*E*8–11-one at a blend ratio of 100 : 5 : 1.

RESULTS AND DISCUSSION

GC-EAD analyses of pheromone gland extract of female WSTM revealed five compounds that consistently elicited responses from male moth antennae (Figure 2). Retention indices on three columns and mass spectra of EAD-active compounds A and C suggested, and GC-MS of authentic standards confirmed, that they were Z6,Z9–21-Hy (A) and Z6–11-one (C). Quantities of compound E were below the detection threshold of both the flame ionization detector (FID) and the mass spectrometer. However, its retention indices on DB-5, DB-210, and DB-23 coated columns were identical to those of previously reported Z6,*E*8–11-one (Gries et al., 1997). Corresponding retention times and comparable antennal activity of synthetic Z6,*E*8–11-one and WSTM-produced E supports structural assignment of Z6,*E*8–11-one for the component *E*. Retention indices (DB-5: 2261; DB-210: 2641; DB-23: 2753) of the second strongest EAD-active compound (D in Figure 2) were indicative of a C_{21} ketone with one or two (nonconjugated) double bonds. GC-MS analyses of a concentrated (> 50 female equivalents) pheromone extract revealed a weak but diagnostic mass spectrum for compound D (Figure 3). The molecular ion *m*/z 309 (M+1) suggested a hene-

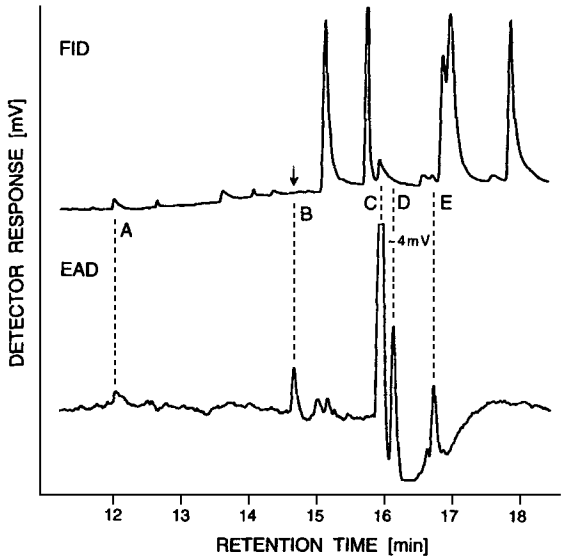

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *O. thyellina* antenna) responses to one female equivalent of pheromone gland extract. Hewlett Packard 5890A gas chromatograph fitted with on-column injection device and equipped with a fused silica column (30 m × 0.25 mm ID) coated with DB-210; temperature program: 50°C for 1 min, 25°/min to 100°C, then 10°/min to 220°C, (hold for 10 min).

icosene ketone, and fragmentation ion *m/z* 197 indicated a keto group in C-9 (Figure 3). Allylic (*E*)-7- or (*Z*)-7 double bonds would have resulted in a distinctively higher retention index, and thus could be excluded as double bond positions. Our hypothesis of a (*Z*)-6 double bond in WSTM-produced D was based on findings that all known keto pheromones in *Orgyia* spp. contain a *Z* double bond in C-6. Synthetic (*Z*)-6-heneicosen-9-one had a mass spectrum consistent with that of WSTM-produced D, retention times corresponding with D on all three columns (DB-5, DB-210, and DB-23), and EAD activity comparable to that of D. Synthetic (*E*)-6-heneicosen-9-one, cochromatographing with Z6–9-one, was only weakly EAD active and had no pheromonal activity in wind-tunnel bioassays (Table 1). Synthetic (*E*)-5- and (*Z*)-5- as well as (*E*)-4- and (*Z*)-4-heneicosen-9-one had different retention times than WSTM-produced D, and thus could be eliminated as candidate pheromone components. EAD-active compound B in pheromone gland extracts (Figure 2) is still unknown.

In field experiments in Japan, pheromone blends with Z6–11-one plus Z6–9-one—the latter ketone termed here "thyellinone"—synergistically attracted

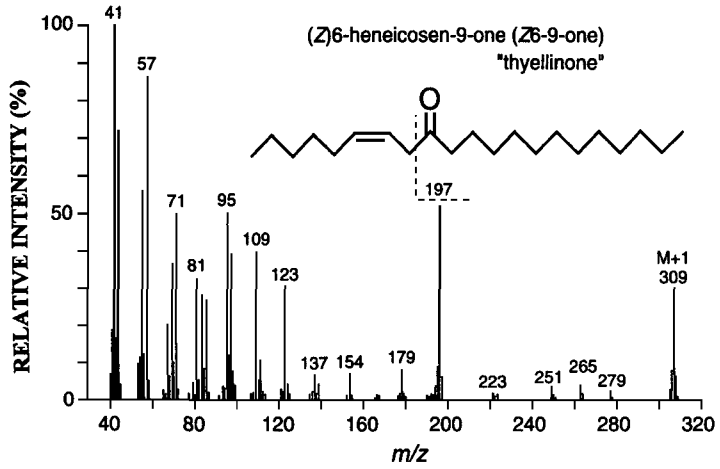

FIG. 3. Full-scan electron impact mass spectrum of (Z)-6-heneicosen-9-one; obtained with a Varian Saturn II Ion Trap GC-MS.

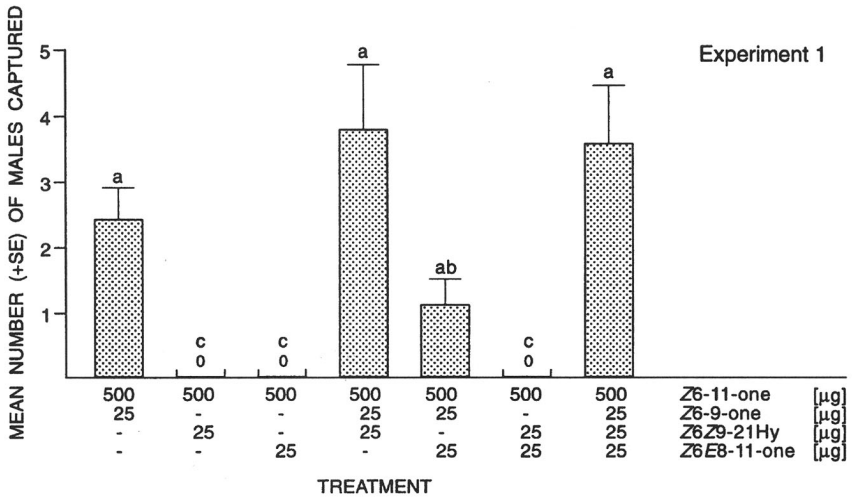

FIG. 4. Mean number of male *O. thyellina* captured in sticky 2-liter Delta milk carton traps baited with (Z)-6-heneicosen-11-one (Z6–11-one) in combinations with (Z)-6-heneicosen-9-one (Z6–9-one), (Z)-6,(E)-8-heneicosadien-11-one (Z6,E8–11-one) and (Z)-6,(Z)-9-heneicosadiene (Z6,Z9–21-Hy); 10 replicates; July 29–August 8, 1997; Morioka, Japan. Bars with the same letter superscript are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) t test, $P < 0.05$] (SAS/STAT, 1988).

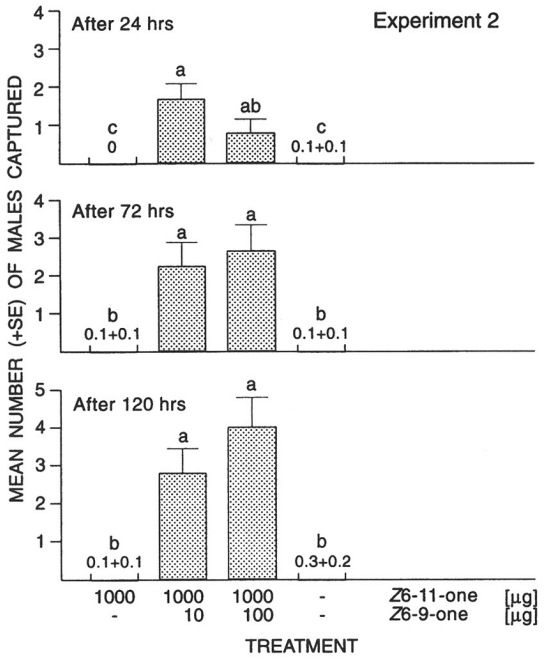

FIG. 5. Mean number of male *O. thyellina* captured in sticky 2-liter Delta milk carton traps baited with (*Z*)-6-heneicosen-11-one (*Z*6–11-one) alone and in combination with (*Z*)-6-heneicosen-9-one (*Z*6–9-one); 10 replicates; July 31–August 5, 1997; Morioka, Japan. Bars with the same letter superscript are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) *t* test, $P < 0.05$] (SAS/STAT, 1988).

WSTM males (Figure 4, experiment 1; Figure 5, experiment 2; Figure 7, experiment 6). Either ketone alone (Figure 7, experiment 6) or volatile blends without thyellinone (Figure 4, experiment 1; Figure 5, experiment 2) failed to attract any male moths. Collectively the data suggest that both *Z*6–11-one and thyellinone are essential pheromone components in the WSTM. This result contrasts with findings in other *Orgyia* spp. for which *Z*6–11-one by itself constitutes an attractive but not species-specific sex pheromone component or sex attractant (Arn et al., 1992). Although admixture of *Z*6,*E*8–11-one to the blend of *Z*6–11-one plus thyellinone did not enhance attractiveness of the pheromone lure in field experiments in Japan (Figure 4, experiment 1; Figure 6, experiments 4 and 5), *Z*6,*E*8–11-one did play a role in wind-tunnel bioassays in New Zealand. In these bioassays, blends with *Z*6,*E*8–11-one as a third volatile component caused significantly more WSTM males to contact the pheromone lure (Table 1). This

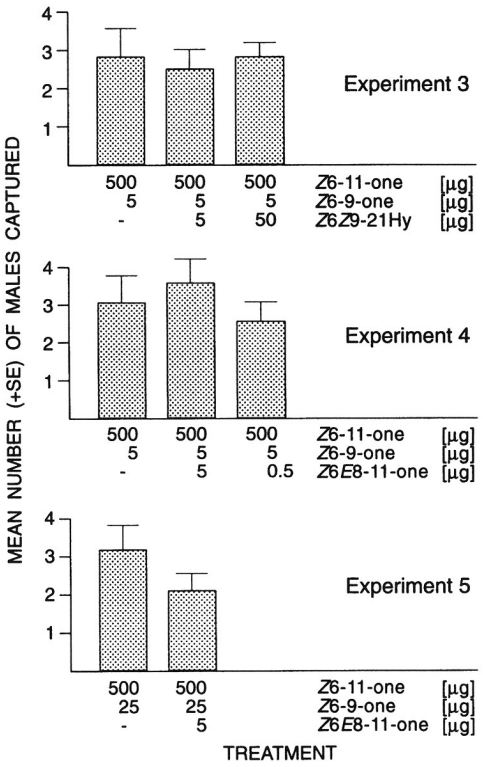

FIG. 6. Mean number of male *O. thyellina* captured in sticky 2-liter Delta milk carton traps baited with (*Z*)-6-heneicosen-11-one (Z6–11-one) plus (*Z*)-6-heneicosen-9-one (Z6–9-one) alone and in combination with either (*Z*)-6,(*Z*)-9-heneicosadiene (Z6,Z9–21-Hy) (experiment 3), or (*Z*)-6,(*E*)-8-heneicosadien-11-one (Z6,E8–11-one) (experiments 4 and 5); 10 replicates for each experiment; August 1–7, 1997 (experiment 3), August 3–8, 1997 (experiment 4), and August 4–8, 1997 (experiment 5); Morioka, Japan; treatments in experiments 3–5 were not significantly different; analysis of variance, followed by comparison of means [Bonferroni (Dunn) *t* test, $P > 0.05$] (SAS/STAT, 1988).

effect may not have been expressed in field experiments, as male moths are captured on tanglefoot already when they enter the milk carton trap or attempt to approach the lure therein. Alternatively, WSTMs that were introduced, reared, and bioassayed in a wind tunnel in New Zealand did not originate from Japan and may indeed use a three-component instead of a two-component pheromone blend.

(*Z*)-6-Heneicosen-9-one (thyellinone) is reported here for the first time as a sex pheromone component in the Lepidoptera and is only one of six pheromone components identified thus far in the genus *Orgyia*. Z6–11-one (Smith et al.,

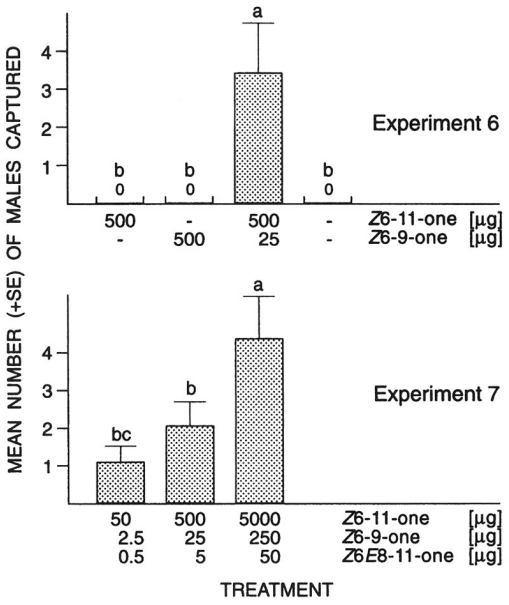

FIG. 7. Mean number of male of *O. thyellina* captured in 2-liter Delta milk carton traps baited with (*Z*)-6-heneicosen-11-one (*Z*6–11-one) and (*Z*)-6-heneicosen-9-one (*Z*6–9-one) singly and in combination (experiment 6; 5 replicates) or baited with a three-component blend [*Z*6–11-one; *Z*6–9-one and (*Z*)-6,(*E*)-8-heneicosavien-11-one (*Z*6,*E*8–11-one)] at increasing doses (experiment 7; 6 replicates); August 4–9, 1997 (experiment 6) and August 6–9, 1997 (experiment 7); Morioka, Japan. Bars with the same letter superscript in experiments 6 or 7 are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) *t* test, $P < 0.05$] (SAS/STAT, 1988).

1975) and *Z*6,*E*8–11-one (Gries et al., 1997) are sex pheromone components of the Douglas-fir tussock moth, *O. pseudotsugata* (McDunnough), and the hydrocarbons *n*-tricosane, *n*-tetracosane, *n*-pentacosane, and *n*-heptacosane in body scales of female white-marked tussock moth (WMTM), *O. leucostigma* (J. E. Smith), release copulatory behavior in conspecific male moths (Grant et al., 1987).

In field-cage, binary choice experiments, traps baited with the commercial lure formulation *Z*6–11-one plus thyellinone captured 8.8 times more male WSTM than those baited with a caged virgin female WSTM [1.4 (mean) + 0.29 (SE) versus 0.16 (mean) + 0.07 (SE) males per trap]. Through the 1998 summer, 45,000 of these synthetic lures were employed in defined areas of Auckland to attempt eradication of a residual WSTM population.

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AGGREGATION AGENTS IN GERMAN COCKROACH *Blattella germanica:* EXAMINATION OF EFFICACY

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Abstract—Aggregation behavior and reduced locomotory activity in the German cockroach is known to be caused by chemical compounds in the feces. The attractive and/or arrestant efficacy of three relevant substances was tested in first instars by a two-choice aggregation test and in adults with a locomotion compensator apparatus that allows quantification of taste-directed orientation and walking speed as a function of antennal stimulation. The three substances tested were a feces crude extract; a mixture of six carboxylic acids (mix G) out of a total of 29 that were identified in the feces extract and tested as single compounds and in various combinations; and a steroid glucoside denoted as blattellastanoside A, which has been suggested as an aggregation arrestant pheromone in *Blattella germanica.* With both of our test methods, feces extract and mix G proved to be very attractive, whereas the effects of blattellastanoside A were, if anything, very poor. Possible reasons for discrepancies are discussed.

Key Words—Dictyoptera, Blattellidae, *Blattella germanica*, German cockroach, aggregation, pheromone.

INTRODUCTION

From thousands of known species, only a small number of synanthropic cockroaches are serious worldwide pests in association with humans. The economically most important species are *Blatta orientalis*, *Periplaneta americana*, and

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Blattella germanica. Of those, the German cockroach, *Blattella germanica* L. (Dictyoptera: Blattellidae), accounts for about 80% of all infestations worldwide. Originating in tropical southeast Asia, it has spread all over the world. From feeding on food stores and by contamination with feces and crop fluid, *B. germanica* is of high economic and medical importance (Brenner, 1995).

Spray-formulated insecticides dominate the market as the preferred method of control (Wickham, 1995), but they do not affect the cockroaches in their hiding places, where they rest during daytime. Furthermore, growing consumer concern about the use of residual pesticides in their living space has caused the pest control industry to make greater use of control methods that minimize pesticide applications. One such alternative might be the use of traps or baits loaded with cockroach pheromones that could attract adults and nymphs or hold them in a certain place.

Three types of pheromones are described as playing a major role in the life of *B. germanica*: (1) Sex pheromones—a two-component contact sex pheromone isolated from the cuticular wax of females that elicits wing-raising in males (Nishida and Fukami, 1983; Nishida et al., 1974, 1976; Schal et al., 1990). Evidence for volatile sex pheromones is reported by Abed et al. (1993) and Liang and Schal (1993). (2) Dispersal pheromones—German cockroach saliva elicits dispersal activity in nymphs and adults (Suto and Kumada, 1981; Nakayama et al., 1984; Ross and Tignor, 1985, 1986). The active dispersion-inducing principle was found in high-molecular-weight proteins of the saliva (Faulde et al., 1989, 1990a,b). (3) Aggregation pheromone(s)—The nature of the so-called aggregation pheromone in *B. germanica* is poorly defined and the literature concerning it somewhat contradictory.

Nymphs and adults of the German cockroach form aggregations in their hiding places. This grouping behavior, combined with reduced locomotion activity, is caused by chemical compounds in the feces. Active components have been extracted from the feces and tested in various behavioral assays (Ishii and Kuwahara 1967, 1968; Kitamura et al., 1974, Wileyto et al., 1984; McFarlane and Alli, 1985; Ross and Tignor, 1986; Sakuma and Fukami, 1985, 1990a,b, 1991; Wendler and Vlatten, 1993; Dambach et al., 1995).

The potential practical use of the aggregation-supporting principles in the feces is described by several authors. Baits with cockroach feces proved to be more attractive in the laboratory to last instars and adults of *B. germanica* and *B. orientalis* than baits without feces (Metzger and Trier, 1975). Furthermore feces extracts reduce the repellency of insecticides in *B. germanica* nymphs and males in the laboratory (Rust and Reierson, 1977a) and increase efficacy in the field (Rust and Reierson, 1977b). Similar effects were shown by Glaser (1980) and Miller et al. (1996). Scharf et al. (1994) reported that sticky traps with a filter paper contaminated by cockroaches were more effective against *B. germanica* than untreated traps.

The development and greater use of aggregation-supporting substances for improvement of cockroach control will require knowledge of the detailed chemical structure of the active components. The so-called aggregation pheromone is supposed to consist of a multi-component mixture of substances divided into a volatile, attractive principle and a nonvolatile, arresting principle. Fuchs et al. (1985) extracted an aggregation-supporting principle from the feces with Freon 11. More than 150 compounds were identified, among them 57 carboxylic acids. After methylation, substantial aggregations were no longer formed. This strongly points to carboxylic acids as components of the aggregation principle.

Attractant components were isolated from frass-contaminated filter paper by Sakuma and Fukami (1990b). The attractant comprised several volatile amines. 1-Dimethylamino-2-methyl-2-propanol was described as the most effective compound, having about 50–1000 times higher activity than the other amines in a linear track olfactometer (Sakuma and Fukami, 1990b). An artificial mixture with this amine was as effective in the olfactometer test as a mixture with 4-(methylsulfinyl)-butanoic acid (Sakuma et al., 1997a). Furthermore alkylamines (especially trimethylamine) were described as components of the attractant principle (Sakuma et al., 1997b).

The isolation of an aggregation-arrestant pheromone was reported by Sakuma and Fukami (1993a,b). A neutral fraction, derived from an extract of frass-contaminated filter paper, showed strong activity in a choice chamber trial. The active principles of this fraction were identified as two glucosyllated steroids after several chromatographic verification steps. These two compounds were named blattellastanoside A and blattellastanoside B. By synthesis of the proposed structures and their derivatives, the exact structure was determined, after two corrections, as 6α-chloro-4β,5-epoxy-5β-stigmastan-3β-yl-β-D-glucopyranoside (blattellastanoside A) and 6α-chloro-4β,5-hydroxy-5β-stigmastan-3β-yl-β-D-glucopyranoside (blattellastanoside B) (Mori and Fukamatsu, 1993, Mori et al., 1993a,b). To the best of our knowledge, this is a novel pheromone structure with no structurally related precedents.

By synthesis of some analogs of blattellastanoside A, it was shown that exchanging the 5β-stigmastan skeleton for 5β-androstane skeletone or the β-D-glucopyranose for D-galactopyranose led to a complete loss of biological activity. A β-cholestan skeleton instead of the original one, and a fluorine substituent instead of the chlorine were biologically active, with the latter being more potent than the natural pheromone (Mori et al., 1996).

As part of a study on the aggregation behavior, we examined the role of an aggregation pheromone in *B. germanica*. Our work encompassed detection of blattelastanoside A from the feces, total synthesis of blattelastanoside A according to a literature procedure published by Mori et al. (1993a,b), and identification of the most attractive/arrestant carboxylic acids from the feces.

All extracts, fractions, and single compounds were tested in a locomotion

compensator test and in a two-choice aggregation test. Both methods have been used recently to study the effect of feces extract on the walking behavior in *B. germanica* (Wendler and Vlatten, 1993) and on the aggregation behavior of nymphs in this species (Dambach et al., 1995).

METHODS AND MATERIALS

Insects and Rearing Procedure. Stock cultures were kept in plastic containers (60 × 43 × 30 cm) at 27 ±2°C and 12L : 12D photoperiod. The cockroaches were fed with commercial dry dog food (Vipromix) and supplied with water ad libitum. New colonies were built up by placing 300 ootheca-bearing females in a cylinder (25 cm diameter) that had a plastic gauze 2 cm above the ground and two openings below. The inner and outer walls of the cylinder were treated with talcum powder and placed in a rearing container. The hatching nymphs fell through the gauze and left the cylinder through the two openings, being unable to reenter the cylinder because of the powdered walls.

Experimental Animals. The nymphal aggregation tests were performed on first instars (L_1, age: 3–4 days after hatching from the oothecae). In order to obtain individuals of known age, isolated from adults and other larval stages, the oothecae were taken from gravid females and kept moist until eclosion (for details see Dambach et al., 1995). The ootheca and the newly hatched nymphs (about 25–35 per ootheca) were kept in a thermostatically controlled chamber at 25°C and 14L : 10D photoperiod. The experimental animals for the locomotion compensator test were taken as adults directly from the colony containers. Care was taken to select individuals of the same size (about 15 mm long) and free of any damage to legs or antennae.

Feces Crude Extract. The solid feces particles were regularly collected from the containers, and all food and insect particles removed. Feces (55 g) from *B. germanica* were mixed with 120 g quartz sand and vigorously ground in a mortar. The ground mixture was transferred to a glass column (3 × 30 cm) and extracted with methanol (3 × 200 ml). The eluate was adjusted to a concentration of 10 mg/ml (referred to solid material) by adding methanol, and yielded 760 ml of a deep red–brown solution (= 7.6 g crude material), which was used as stock solution for both test systems, locomotion compensator test, and aggregation test.

Analysis of Blattellastanoside A in Feces Crude Extract. The content of blattellastanoside A in the feces crude extract was determined by LC-MS with selected ion monitoring of the formate adduct $(M\ HCCOO)^-$ in electrospray negative ion mode. HPLC-MS was performed on a Finnigan TSQ 7000 system equipped with a Chait type electrospray source (heated metal capillary) coupled with a HPLC-System, Applied Biosystems ABI 140 B HPLC pump, Hewlett

Packard HP 1050 Autosampler, Applied Biosystems UV-detector and a Spark Mistral column oven (gradient: A: water + 25 mmol/liter ammonium formate, B: methanol/water 9 : 1 + 25 mmol/liter ammonium formate, 0 min: 30% B, 3 min: 30% B, 63 min: 90% B, 70 min: 90% B; flow: 200 μ/min; colunn: 125 × 2, nucleosil C_{18}, 120 Å, 5 μm; injection volume: 5 or 10 μl; column oven: 40°C). Retention time and response factor were determined by injection of authentic blattellastanoside A.

Identification of Carboxylic Acids in Cockroach Feces Crude Extract. For the determination of the chemical nature of the active compounds, a second batch of feces crude extract was prepared from 20 g feces as described above. The methanolic solution was concentrated to 15 ml. After the addition of 75 ml of 5% aqueous hydrochloric acid, the solution was extracted with diethylether (3 × 50 ml). The aqueous layer was adjusted to pH 12 by addition of 5% aqueous sodium hydroxide and subsequently extracted with diethylether (3 × 100 ml). This diethylether solution contained the basic components. The diethylether layer from the extraction of the hydrochloric acid solution was extracted with 5% aqueous sodium hydroxide (3 × 50 ml), leaving the neutral components in the diethylether solution. The combined aqueous sodium hydroxide layers were adjusted to pH 10 with 5% aqueous hydrochloric acid and extracted with diethylether (3 × 100 ml). This diethylether solution contained the phenolic components. The aqueous layer was adjusted to pH 1 and subsequently extracted with diethylether (3 × 100 ml). This diethylether solution contained the acidic components. All diethylether solutions were concentrated to 10 ml. Only the solution with the acid components was found to have aggregation values as high as the feces crude extract. The solutions of the other components produced significantly lower values. The acidic components solution was analyzed by GC-MS on a 5890 Hewlett Packard gas chromatograph coupled with a Finnigan TSQ 700 mass spectrometer (capillary column: DB-1, 60 m, 0.32 mm, 0.25 μm; temperature program: 70°C for 3 min, 70°C to 320°C at 5°C/min, hold at 320°C (for 10 min); carrier gas: helium; injector: split/splitless, 250°C; injection volume: 1 μl; scan range: 35–800 amu, transfer line: 280°C. The samples were treated before injection with *N*-methyl-*N*-trimethylsilyl trifluoracetamide for trimethylsilylation of the carboxylic acids. Mass spectra and retention times were compared with authentic carboxylic acids.

Synthesis of Blattelastanoside A. Commercially available stigmasterol was chosen as the starting material and converted to β-sitosterol via a standard procedure (Steele and Mosettig, 1963) in a five-step synthesis. The further transformations were performed with minor modifications according to the synthesis by Mori and Fukamatsu (1993) and Mori et al. (1993a,b). The synthetic blattellastanoside A was completely identical to the reported compound with respect to all spectroscopic data. FAB-MS m/z (%) = 649 ($M+Na^+$, 77), 627 ($M+H^+$, 5), 465 (aglycone + H^+, 12), 447 (aglycone − H_2O + H^+, 60), 429 (aglycone − Cl

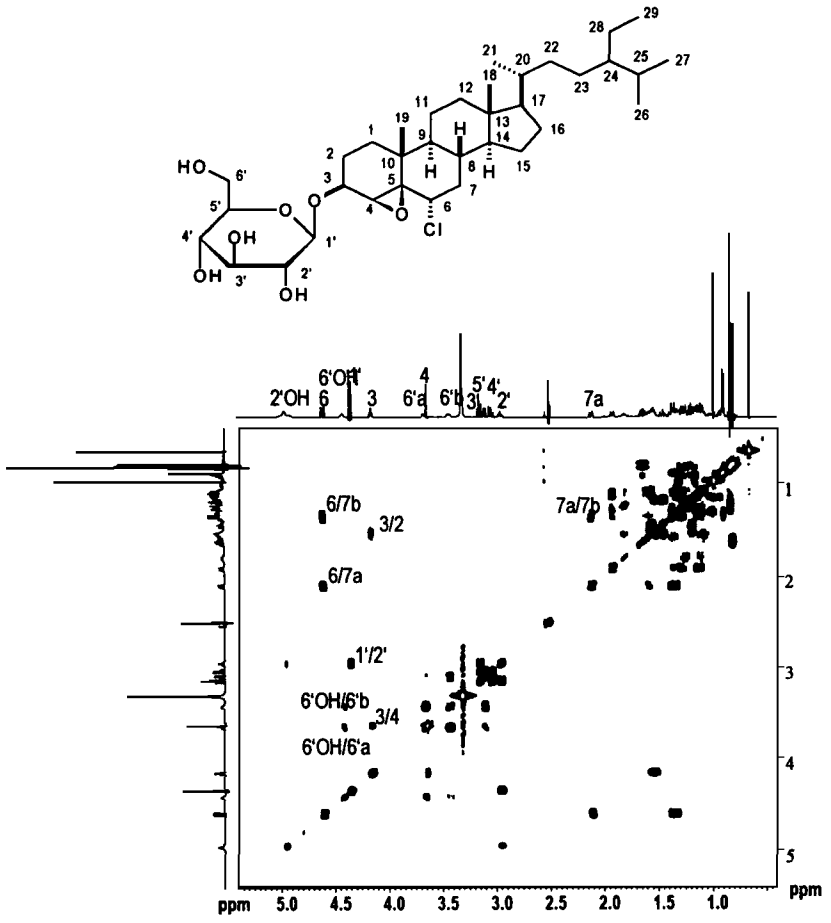

FIG. 1. Structure of blattellastanoside A, synthesized according to Mori and Fukamatsu (1993) and Mori et al. (1993a,b) and ^1H, ^1H-COSY spectrum with signal assignment (500 MHz DMSO).

+ H^+, 100), 411 (aglycone – H_2O – HCl + H^+, 65), 393 (aglycone – C_4H_7OH + H^+, 38) (Figure 1).

Blattellastanoside A Test Solution. Blattellastanoside A was dissolved in methanol. The concentration of the stock solution was 1 mg/ml for the locomotion compensator test and 200 μg/ml for the aggregation test.

Nymphal Aggregation Test. The assay we used was a two-choice test in which the L_1 nymphs had to choose between a test and a control site (Figure 2A). On the bottom of a glass cylinder (7 cm diam., 9 cm high) two strips of

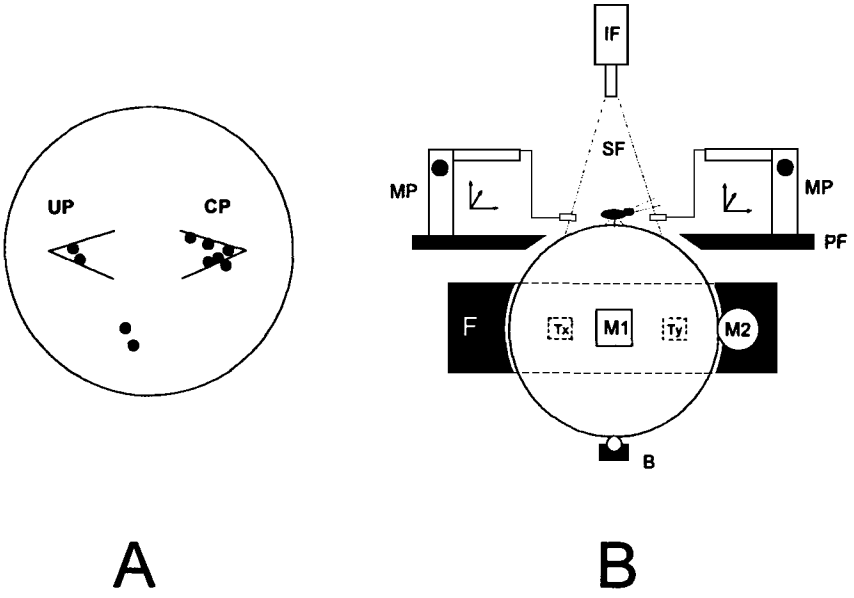

FIG. 2. Methods used to test the efficacy of aggregation agents. (A) Nymphal aggregation test situation with two folded filter papers. CP, contaminated paper (= test site); UP, uncontaminated paper (control site). The example of nymph distribution (dots) illustrated would result in an aggregation index (AI) of +0.4. (B) Locomotion compensator test (schematic view). Sphere diameter 50 cm. IF, infrared light source and scanning camera. SF, scanning field of the infrared scanning device. M1, M2, orthogonally adjusted motors with rubber coated wheels. Tx, Ty, optical slit discs for measurement of movements. MP, manipulators for positioning of the paper stimulus source. PF, platform. B, ball bearing. F, frame holding the motors and slit discs.

filter paper (1 × 3 cm) were placed, folded into open arrowheads, and arranged with the open sides facing each other. One strip was impregnated with the test substance while the control strip was soaked with the solvent (methanol) alone, and both were allowed to dry. For each test a sample of 10 L_1 was used, and their distribution between the two sites was noted after 1 hr. The tests were repeated nine times with new L_1 each time. Thus, for each of the tested substances and concentrations, counts of 10 replicates were available.

For evaluation a measure of distribution was used [aggregation index (AI)] which expresses the degree of preference for one of the two alternative sites: $AI = (N_{CP} - N_{UP}/N_T$; where N_{CP} is the number of individuals on contaminated paper (= test paper), N_{UP} is the number of individuals on uncontaminated paper (= control paper), and N_T is the total number of animals (= N_{CP} + N_{UP} + free

animals). The formula gives values between −1 (all animals on the control strip) and +1 (all on the test strip). Positive signs therefore indicate arrestant effects of the test substance and negative mean repellency. If all individuals were evenly distributed between the two strips (5 : 5) or if all were beside them in the vessel, a value of 0 would result. In earlier studies (Dambach et al., 1995), a factor of 10 was used in the formula in order to obtain full numbers between −10 and +10. Here we favor a shortened version to obtain values in the same order and signs corresponding to those used for the taste-directed component (TDC) in the locomotion test.

Locomotion Compensator Test Experimental Apparatus. The cockroaches were tested on a locomotion compensator, the operating principle of which has been described in detail by Wendler and Vlatten (1993). In short, the animals walk on top of a sphere (Figure 2B). Translatory movements of the animal were measured by an infrared scanning device (IF) which controls two motors (M1, M2) that rotate the sphere in such a way that all translatory movements are compensated. The unrestrained cockroach was thereby held on top of the sphere, nevertheless being able to choose any walking direction and speed. The behavior of the animal was monitored by an infrared-sensitive video camera. The rotations of the sphere, representing the movements of the animal, were measured with a time resolution of 0.2 msec and a positional precision of 0.5 mm.

The animals were presented with two filter paper strips from opposite sides (Figure 2B). The strips measured 0.5 × 1 cm and were affixed on their edges to the manipulator, which itself stood permanently on a platform. The narrow side of the strip was presented to the animal at a height of 1 cm, the leading edge being positioned within reach of the tips of the antennae when the animal was oriented towards the strip. The test strip was soaked with 10 μl of the test substance, the control strip with 10 μl methanol. Both were allowed to dry. Tests were performed with adult males in darkness at a temperature of 23 ±3°C and at different times of day. Between 13 and 15 animals were tested for each situation.

Evaluation. We determined the direction, straightness, and taste-directed component of the runs as well as the speed of walking. These were the parameters used in earlier studies to characterize the walking behavior in the German cockroach (Wendler and Vlatten, 1993). Measurements were taken at 0.2-sec intervals.

The direction and straightness of a run (Figure 3A) are referred to as its vector (Figure 3B). Its direction α is the direction from the starting point to the end point of a run. Its length (VL) is the distance (shortest connection) between the two points divided by the length of the actual track. Vector length is solely a measure of the straightness of the track. To obtain a quantitative estimate of the accuracy with which the animal walks towards a filter paper strip soaked in a tasty or attractive substance, the taste-directed component is determined as $TDC = VL \times \cos(\alpha - \alpha_0)$, $0 < VL < 1$; $\alpha_1 = 0^0$ = position of the strip. This

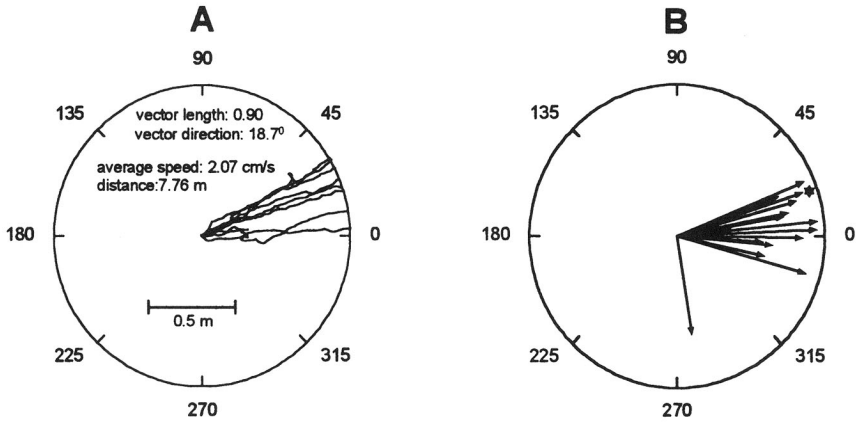

FIG. 3. (A) Example run, lasting 6.3 min. The paper strip with feces extract was located at 0°, the control at 180°. When the track of the continuous run reaches a certain distance from the center, the pen is reset to the center. In the vector diagram (B), this example run results in the vector marked by an asterisk. (B) All runs with the undiluted feces extract, resulting in a TDC of 0.71 (see text and Figure 4B).

corresponds to the "homeward component" established for pigeons (Batschelet, 1981). It takes into account not only the straightness but also the cosine of the angle of deviation from the signal source. $TDC = +1$ characterizes a perfectly straight run towards the strip with the test substance, and $TDC = -1$ a straight run away from the substance and towards the control strip. Means are presented with standard errors.

RESULTS

Carboxylic Acids. The active feces crude extract was separated by acid–base, liquid–liquid partition. The fraction with the acidic components gave the highest aggregation values, i.e., as high as the feces crude extract. This fraction was therefore analyzed by GC-IMS techniques and 29 carboxylic acids were identified (Table 1).

These 29 carboxylic acids were tested in both test systems first as single compounds and their effectiveness was compared with that of the total mixture at equal weight compounds. As none of the single compounds reached the effectiveness of the whole mixture, various combinations and numbers of components were prepared and tested. As a result, the mixture of the six carboxylic acids that we have described below as mix G was found to be most effective.

Mixture of Carboxylic Acids (Mix G). Equal weight amounts of commer-

TABLE 1. CARBOXYLIC ACIDS IDENTIFIED IN FECES EXTRACT OF *Blattella germanica*

Butyric acid[a]	3-Phenylpropionic acid[a]
3-Methylbutyric acid[a]	3-(*m*-Hydroxyphenyl)propionic acid
Valeric acid[a]	3-(*p*-Hydroxyphenyl)propionic acid
Caproic acid[a]	Anthranilic acid
Oenanthic acid	Lactic acid
Myristic acid[a]	3-Phenyllactic acid
Palmitic acid[a]	Fumaric acid
Stearic acid[a]	Glutaric acid
Oleic acid[a]	Adipic acid
Elaidic acid	3-Methyladipic acid
Cyclohexanecarboxylic acid[a]	Pimelic acid
Benzoic acid[a]	Suberic acid
Phenylacetic acid[a]	Azelaic acid
p-Hydroxyphenylacetic acid	Sebacic acid
	1,12-Dodecanedioic acid

[a]Carboxylic acids were also identified by Fuchs et al. (1985).

cially available 3-phenylpropionic acid, oenanthic acid, myristic acid, cyclohexanecarboxylic acid, sebacic acid, and DL-3-phenyllactic acid (all from Merck, except for DL-3-phenyllactic acid from Fluka) were dissolved in methanol. The concentration of the stock solution was 5 mg mixture/ml for the locomotion compensator test and 1 mg mixture/ml for the aggregation test.

Determination of Concentration of Blattellastanoside A in Feces Crude Extract. The feces crude extract was analyzed by quantitative HPLC-MS techniques. Blatellastanoside A could not be detected in the feces extract within the detection limit (0.5 ppm) of the method, which is approximately 20-fold below the concentration of blattellastanoside A reported by Sakuma and Fukami (1993b) (15.0 mg/1.373 g $\cong$ 11 ppm).

Locomotion Compensator Test. We compared the attractiveness of feces extract, blattellastanoside A, and mix G. When feces extract was presented, the cockroaches walked towards the filter paper with the feces extract and neglected the strip with the control (Figures 3 and 4B). They stayed in contact with the test strip almost continuously. As could be seen with the help of an infrared video system, the contact was often maintained by only one antenna, and changes between right and left antenna were often observed. The attractiveness diminished with higher degrees of dilution. Blattelastanoside A had no attractive or arrestive effect whatsoever. Mix G proved to be attractive at lower concentrations. None of the three test substances had a repellent effect.

Nymphal Aggregation Test. With this test we examined and compared the efficacy (i.e., the potency as an arrestant) of mix G, blattellastanoside A, and feces extract, each at four concentrations (Figure 4). Feces extract in the initial concentration gave the highest aggregation values, and the efficacy diminished

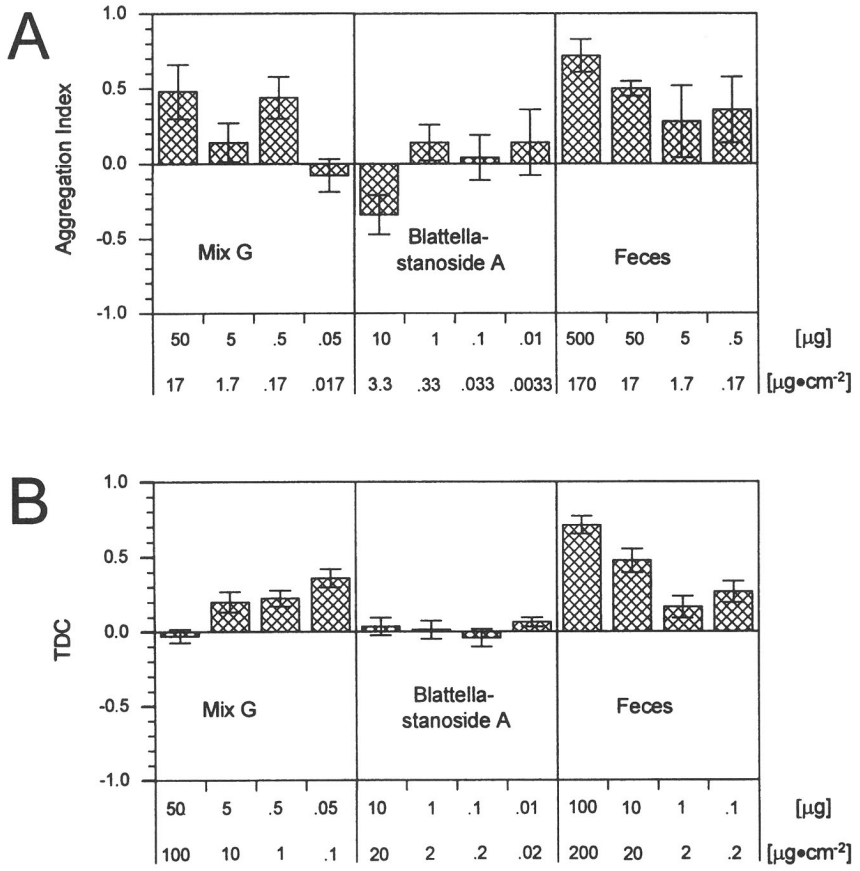

FIG. 4. Comparison of attractiveness of mix G, blattellastanoside A, and feces extract ascertained in the nymphal aggregation test (A) and the locomotion compensator test (B). Concentration of the test substances is given in amount of substance per test filter paper strip and per paper strip area. TCD taste directed component. Means are given with standard error.

with higher dilutions. Mix G was also effective except at the lowest concentration. Blattellastanoside A in the highest concentration acted as a repellent, and with further dilution its arrestant effect was, if anything, very poor.

DISCUSSION

The aggregation pheromone is thought to consist of a volatile (attractant) and a nonvolatile (arrestant) principle. In both the two choice aggregation test

with L_1 nymphs and the locomotion compensator test, the effect of arrestant components is detected, as in the choice chamber assay of Sakuma and Fukami (1991). However, as in their assay, an initial influence of volatile substances cannot be excluded, since the test systems do not determine whether the animals find the filter paper with the test substance by chance or deliberately.

Sakuma and Fukami (1991) showed in their experiments with gauze-covered papers that an initial arrestment due to volatile substances declined within 20 min. Since we evaluated the nymphal aggregation test after 1 hr, we are convinced that the results represent the effect of arrestant components.

In the locomotion compensator test, the behavioral effect of volatile substances is analyzed one step further, since the normal distance loop is opened, leading to a Sisyphus situation for the animal. The infrared video camera revealed that antennal contact with the paper strip is essential for the animals. Once the animals had made contact with the test strip, the contact was maintained, depending on the substance and its concentration. In order to keep this contact, the cockroaches need to walk on the locomotion compensator. The primary effect of the arrestant substance is therefore to gain a maximum of contact between antennae and the substance. This leads to a stop in the normal closed loop situation.

With feces extract, the two methods produced identical results. Positive results were also obtained with the mixture of carboxylic acids (mix G). However, in the nymphal test the effect of mix G was restricted to lower concentrations, while in the locomotion compensator test, the TDC increased with higher concentrations. One possible explanation for the fact that the aggregation index does not increase or decrease in a harmonic way when mix G is diluted may be that the components of the mixture have their optimal effects at different dilutions. Therefore the compound may produce such a result, either by simple superposition of the individual effects or even by nonlinear interactions at the receptor level or at the neuronal level. The different results with the two test systems may be explained by the fact that mix G, in contrast to the feces extract, contains only a few components, to which nymphs seem to respond in a different way than adults. With blattellastanoside A we expected that the results of the two methods would match or at least be similar. To our surprise, however, blattellastanoside A produced no attractive or arrestant effect whatsoever. In the nymphal test, it even had a repellent effect at high concentrations.

The reason for the discrepancy between our findings and those of Sakuma and Fukami (1993a,b) may lie in the different test systems used. On the other hand, both our test systems, the nymphal test and the locomotion compensator test, are valid as the attractiveness of feces extract proves. We conclude that the arrestant component of the feces is not blattellastanoside A.

To confirm that the extract of the feces from our stock did indeed contain blattellastanoside A, we analyzed the feces extract by HPLC-MS techniques. We

found no blattellastanoside A in a concentration higher than 0.5 ppm, whereas Sakuma and Fukami (1993b) found a content of 15.0 mg/1.373 g crude extract (≅11 ppm, referred to solid material). This discrepancy may result from the different methods used. Sakuma and Fukami (1993b) prepared the feces crude extract by extraction of feces-soaked filter paper. Contaminant components from the filter paper might explain the discrepancy. Because we find no effect of blattellastanoside A on the aggregation behavior as tested in our two test systems, and because we find a significant effect of feces-originated carboxylic acids, and the active feces extract from our population does not contain blattellastanoside A in the cited concentration, we have drawn the conclusion that blattellastanoside A is not as important for the aggregation behavior in *Blattella germanica* as we expected. Since the tests were performed on different stocks of *Blattella germanica* in Japan and in Germany, differences in the populations are also feasible.

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SYNERGISTIC EFFECT OF ETHANOL TO α-PINENE IN PRIMARY ATTRACTION OF THE LARGER PINE SHOOT BEETLE, *Tomicus piniperda*

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Abstract—α-Pinene and ethanol were released in the approximate proportions 1:0.1, 1:0.9 and 1:9 (at 21°C). Ethanol, released in the range of 3–279 mg/day, generally synergized the attraction of *T. piniperda* to α-pinene (30 mg/day at 21°C), although attraction to the mixtures varied within and between years. The low release rate of ethanol together with α-pinene attracted a significantly higher number of beetles than α-pinene alone in 1995, April of 1996, and in 1997. Lures with the medium release rate of ethanol were the most attractive only in March of 1996. The high dose of ethanol significantly synergized attraction to α-pinene in 1995 and 1997. The variable attraction of *T. pinipeda* to ethanol and α-pinene at various release rates and proportions may be due to the temperature dependent nature of beetle antennal sensitivity. At ambient temperatures of 10–13°C. *T. piniperda* was most attracted to the lures with α-pinene and high release rates of ethanol, at 14–17°C it was most attracted to those with medium release rates of ethanol, and at 18°C and higher it was most attraacted to those with low release rates of ethanol.

Key Words—*Tomicus piniperda*, synergism, α-pinene, ethanol, ambient air temperature, release rates.

INTRODUCTION

The pine shoot beetle, *Tomicus piniperda* (L.) (Coleoptera, Scolytidae) is one of the most destructive insect pests affecting pines in its native range of Europe and Asia (Långström, 1980; Ye, 1991; Långström and Hellqvist, 1993). Accidentally

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introduced to North America, it was first discovered near Cleveland, Ohio, in July 1992 (Haack et al., 1997). Surveys in 1997 showed that eight states in the Great Lakes region and southern Ontario were infested (Haack et al., 1997; NAPIS, 1998).

The strong attraction of *T. piniperda* to Scots pine odors is well documented (Perttunen et al., 1970; Oksanen et al., 1971; Byers et al., 1985; Klimetzek et al., 1986; Vité et al., 1986; Schroeder and Eidmann, 1987; Zumr, 1989; Byers, 1992). Although emission of secondary attractants by females has been reported by some authors (Schönherr, 1972; Carlé, 1974; Francke and Heemann, 1976), others report that secondary attraction is unlikely (Byers et al.,1 985; Löyttyniemi et al., 1988).

Tomicus piniperda reproduces in recently cut, windbroken, windthrown, or otherwise severely weakened pines. These damaged or weakened trees emit monoterpenes (Ikeda et al., 1980; Strömvall and Petersson, 1991), as well as ethanol, which forms as a result of degradation processes in the tissue of hosts suitable for colonization by *T. piniperda* (Moeck, 1970; Ikeda et al., 1980; Sjödin et al., 1989). Ethanol has been reported to both synergize (Vité et al., 1986; Klimetzek et al., 1986; Schroeder and Eidmann, 1987; Schroeder and Lindelöw, 1989; Zumr, 1989; Byers, 1992) and inhibit (Klimetzek et al., 1986; Schroeder, 1988) attraction of *T. piniperda* to monoterpenes. Vité et al. (1986) reported approximately an eightfold increase in trap catches when baited with ethanol and the Scots pine monoterpenes α-pinene and terpinolene compared to that of monoterpenes alone. Schroeder and Eidmann (1987) reported that *T. piniperda* was attracted to and attacked healthy Scots pines baited with ethanol. Schroeder and Lindelöw (1989) found that attraction of *T. piniperda* to α-pinene is synergized by ethanol at low release rates of α-pinene, but very high release rates of α-pinene caught more beetles than those combined with ethanol. Zumr (1989) reported increasing attraction with increasing release rates of ethanol together with α-pinene (tested in the proportions of 1 : 1, 1 : 3, 1 : 5, and 1 : 7). Byers (1992) reported that ethanol released at increasing rates (0.08–800 mg/day) does not affect *T. piniperda* attraction to the Scots pine monoterpenes α-pinene, Δ-3-carene, and terpinolene released at rates of 28, 6, and 2.5 mg/day, respectively, but ethanol increased attraction when tested together with lower release rates of monoterpenes (3.3% of the higher rates). However, Klimetzek et al. (1973) reported that ethanol released at 24 mg/day synergizes the attractiveness of *T. piniperda* to α-pinene and terpinolene, and attraction decreases witih increasing release rates of ethanol. Schroeder (1988) reported that ethanol released at rates from 36 mg/day to 50 g/day inhibit the attractiveness of *T. piniperda* to α-pinene released at 240 mg/day.

Such contradictory results are difficult to interpret. The response of *T. piniperda* to different amounts of ethanol and monoterpenes may be affected by ambient temperatures during different field experiments. Temperature has

been reported to affect chemically mediated communication in insects (Cardé and Roelofs, 1973; Comeau et al., 1976; Castrovillo and Cardé, 1979; Linn et al., 1988, 1991; Bento et al., 1993; Charlton et al., 1993; Facundo et al., 1994). Moreover, Bestmann and Dippold (1983) reported that moth antennal response varied at different temperatures with a constant pheromone stimulus.

In this study the synergistic effect of ethanol on *T. piniperda* attraction to α-pinene was examined. The trap catch data were also correlated with the ambient temperatures during *T. piniperda* reproductive flight.

METHODS AND MATERIALS

Experiments were conducted in a 35-year-old Scots pine stand 5 km east of Lockport, Niagara County, New York in 1995, 1996, and 1997. This 4.8-ha unmanaged stand has been damaged by *T. piniperda* for more than a decade (Czokajlo et al., 1997). No trees were harvested in the stand for several years prior to this study, and all freshly snow-damaged trees were removed prior to *T. piniperda* reproductive flight to avoid competition from natural sources.

Beetles were caught in 36, 8-unit multiple-funnel traps (Lindgren, 1983) spaced 15 m or more, and hung with the collection cup ca. 30 cm above the ground. The chemicals used were α-pinene (Aldrich, 98% $[\alpha]^{22}$-0°) and 95% ethanol. The compounds were released separately from 2-ml glass septum vials through glass capillaries inserted into the vial septum or from uncapped vials. There were six treatments: (1) blank trap, (2) α-pinene (30 mg/day at 21°C), (3) ethanol (28 mg/day at 21°C), and (4–6) α-pinene (30 mg/day at 21°C) + ethanol in the proportions of 1 : 0.1, 1 : 0.9, and 1 : 9 (small, medium, and high release rates of ethanol), respectively. Each treatment was replicated six times. Traps were emptied and lures refilled weekly. The release rates of α-pinene and ethanol were estimated gravimetrically in the laboratory at the constant temperatures of 10, 15.5, and 21°C.

Data from all field tests were separately subjected to single factor ANOVA. This analysis was performed on two data sets: (1) pooled among and within each year all α-pinene + ethanol treatments to test for overall synergism of ethanol to α-pinene, and (2) seasonal data sets to test for synergism of different release rates of ethanol in *T. piniperda* attraction to α-pinene. Data from laboratory estimated release rates and proportions of α-pinene and ethanol were subjected to two-way ANOVA with replication. All data were transformed to satisfy ANOVA assumptions as follows: pooled over year trap catches were log-transformed, trap catches from 1995 were rank-transformed, trap catches from March of 1996 were log-transformed, and trap catches from April of 1996 and 1997 were converted to the proportions of the catch of each replicate and then log-transformed.

The laboratory estimated release rates of α-pinene and ethanol were log-transformed and their proportion square root-transformed. The LSD test was used to compare means (Stat Soft, Inc., 1995).

RESULTS

The reproductive flight activity of *T. piniperda* during experimental years and corresponding maximum daily temperatures are given in Table 1.

Synergism of Ethanol to α-Pinene. Ethanol, released in the range of 3–279 mg/day (21°C) (Table 2), had a synergistic effect on the attraction of *T. piniperda* to α-pinene (30 mg/day at 21°C) (LSD, $P = 0.011$). In 1995 and 1997, pooled captures in traps baited with α-pinene + all release rates of ethanol were significantly higher than those of α-pinene alone (LSD, $P = 0.009$ and $P = 0.017$, respectively). Captures in traps baited with α-pinene + the three release rates of ethanol from both 1996 data sets were not significantly different from those of α-pinene alone.

Dose Effect of Ethanol. In 1995, significantly more beetles were attracted to the traps baited with α-pinene and low (3 mg/day at 21°C) and high (142 mg/day at 10°C) (Table 2) release rates of ethanol than to the traps baited with α-pinene alone (LSD, $P = 0.003$ and $P = 0.043$; Figure 1). Captures in traps baited with α-pinene and ethanol at a medium release rate were slightly higher but not significantly different from those of α-pinene alone. In March of 1996, captures in traps baited with α-pinene and the medium release rate of ethanol (19 mg/day at 15.5°C) were significantly higher than those baited with α-pinene alone (LSD, $P = 0.049$). Significantly fewer beetles were captured in traps baited

TABLE 1. MAXIMUM DAILY TEMPERATURES DURING REPRODUCTIVE FLIGHT OF *T. piniperda* NEAR LOCKPORT, NIAGARA COUNTY, NEW YORK IN 1995–1997

Flight season	Dates of flight	Maximum daily temperatures (°C)[a]
1995	Mar 15–20	21, 18, 12, 10, 10, 21
March 1996[b]	Mar 31–Apr 1	17, 15
April 1996[b]	Apr 11, 15–17	18, 19, 18, 17
1997	Mar 28–Apr 3	24, 16, 7, 1, 12, 18, 18

[a]Temperature data for 1995 and 1996 were obtained from the Climatological Station, Lockport 2NE located 5 km west of the experimental site (NOAA, 1995, 1996), and for 1997 recorded in a nearby stand on Omnidata Pod Model DP220 (Omnidata International, Inc., Logan, UT) (Knodel and Barak, 1997).

[b]In 1996 *T. piniperda* had two reproductive flight peaks that were separated by nine days of cold weather.

TABLE 2. ATTRACTION OF *Tomicus piniperda* TO TRAPS BAITED WITH α-PINENE AND ETHANOL AND RELATIONSHIP TO AMBIENT TEMPERATURE NEAR LOCKPORT, NIAGARA COUNTY, NEW YORK IN 1995–1997

Year	Temp. range (No. of days)[a]	Percent of beetles captured[b] with α-pinene (30 mg/day–ethanol in lure		
		1 : 0.1	1 : 0.9	1 : 9
1995	A(3); C(3)	54[c]	11	35[c]
Mar. 1996	B(3)	9	65[c]	26
Apr. 1996	C(4)	61[c]	16	23
1997	A(1); B(1); C(3)	36[c]	21	43[c]

[a]Temperature ranges are as follows: (A) 10–13°C, (B) 14–17°C, (C) 18°C and higher.
[b]Captures are expressed as percent of total captures in traps baited with ethanol and α-pinene for a given year.
[c]Captures differ significantly from those of α-pinene alone.

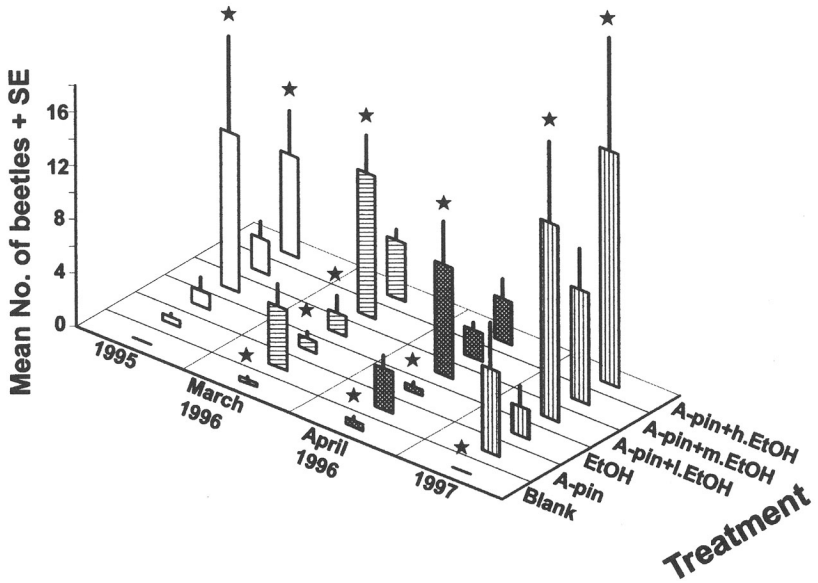

FIG. 1. Mean number of *Tomicus piniperda* caught in Lindgren funnel traps baited with α-pinene (A-pin, 30 mg/day at 21°C), ethanol (EtOH, 28 mg/day at 21°C), A-pin + l.EtOH, A-pin + m.EtOH, and A-pin + h. EtOH α-pinene (30 mg/day at 21°C) with ethanol in the release rate proportions 1 : 0.1, 1 : 0.9 and 1 : 9, respectively, near Lockport, New York. Stars indicate catches significantly different from that of α-pinene alone (A-pin) (LSD, $N = 6$, $P < 0.05$).

TABLE 3. RELEASE RATES OF α-PINENE AND RELEASE PROPORTIONS OF α-PINENE AND ETHANOL DETERMINED IN LABORATORY AT DIFFERENT TEMPERATURES ($N = 36$)[a]

Temp. (°C)	Release of α-pinene (mg/day)[b]	Proportion of ethanol in lure (±SD) at release rate		
		Low	Medium	High
10	15.25	0.11 (0.81)	0.88 (2.98)	9.25 (34.54)
15.5	20.17	0.12 (1.45)	0.95 (3.69)	9.28 (29.14)
21	30.79	0.09 (0.57)	0.89 (4.93)	9.02 (32.21)

[a]Proportions among release rates of α-pinene and three release rates of ethanol are not modified by changes in ambient temperature ($P = 0.99$).
[b]For each temperature the quantity of α-pinene corresponds to the proportion 1 in the lure composition.

with α-pinene and the low release rate of ethanol than to α-pinene alone (LSD, $P = 0.036$). Captures in traps baited with α-pinene and the high release rate of ethanol were not different from those of α-pinene alone. In April of 1996, only captures in traps baited with α-pinene and low release rates of ethanol (3 mg/day at 21°C) were significantly higher than those of α-pinene alone (LSD, $P = 0.046$). Attraction to the traps baited with α-pinene and medium and high release rates of ethanol was not different from that to α-pinene alone. In 1997, significantly more beetles were captured in traps baited with α-pinene and low (3 mg/day 21°C) and high (142 mg/day 10°C) release rates of ethanol than to the traps baited with α-pinene alone (LSD, $P = 0.016$ and $P = 0.023$, respectively). Captures in traps baited with α-pinene and the medium release rates of ethanol were not different from those baited with α-pinene alone.

Release Rates and Proportions of α-Pinene and Ethanol. Release rates of α-pinene and ethanol varied among tested temperatures but their proportions remained unchanged (1 : 0.1, 1 : 0.9, and 1 : 9) (LSD, $P > 0.3$) (Tables 3 and 4). Proportions between release rates of α-pinene and three release rates of ethanol

TABLE 4. ANOVA TABLE FOR ANALYSIS OF RELEASE PROPORTIONS OF α-PINENE AND ETHANOL ESTIMATED IN LABORATORY UNDER CONSTANT TEMPERATURES OF 10, 15.5, AND 21°C

	df effect	*MS* effect	*df* error	*MS* error	*F*	*P* level
Release proportion	2	216.3	315	0.04	6064.8	0
Temperature	2	0.04	315	0.04	1.15	0.31
Rel. prop. × temp.	4	0.003	315	0.04	0.09	0.99

TABLE 5. ANOVA TABLE FOR ANALYSIS OF RELEASE RATES OF ETHANOL ESTIMATED IN LABORATORY UNDER CONSTANT TEMPERATURES OF 10, 15.5, AND 21°C

	df effect	*MS* effect	*df* error	*MS* error	*F*	*P* level
Release rate	2	645.6	369	0.12	5384.1	0
Temperature	2	14.2	369	0.12	118.1	0
Rel. rate × temp.	4	0.1	369	0.12	0.88	0.47

are not modified by changes in ambient temperature (Table 4). Release rates of ethanol at 10, 15.5, and 21°C were different (LSD, $P < 0.001$ for all) among the three tested levels regardless of temperature (Table 5). Release rates of α-pinene and medium release rates of ethanol were not significantly different within each tested temperature (LSD, $P > 0.05$).

DISCUSSION

Ethanol plays an important role in the host searching behavior of *T. piniperda.* Various release rates of ethanol synergized attractiveness of α-pinene to the beetle. Attraction to the mixture varied among and within years: the low release rate of ethanol toghether with α-pinene attracted a higher number of beetles than α-pinene alone in 1995, April of 1996 and 1997. The medium release rate of ethanol was the most attractive only in March of 1996, and the high release rate of ethanol synergized attraction to α-pinene in 1995 and 1997.

The role of ethanol in the chemical communication of *T. piniperda* has been the focus of numerous studies (Klimetzek et al., 1986; Vité et al., 1986; Schroeder and Eidmann, 1987; Schroeder, 1988; Schroeder and Lindelöw, 1989; Zumr, 1989; Byers, 1992). High release rates of ethanol have been reported to be both increasingly attractive (Zumr, 1989; Byers, 1992), and inhibitory (Klimetzek et al., 1986; Schroeder, 1988). Inhibition obtained by Schroeder (1988) resulted from release rates of ethanol and α-pinene higher than those observed in nature (more than 3 g/day and 240 mg/day, respectively) (Moeck, 1970; Ikeda et al., 1980; Byers et al., 1985). In our study, synergism of ethanol to α-pinene was similar to that of Vité et al. (1986) and Schroeder and Lindelöw (1989).

Changes in ambient temperature may affect *T. piniperda* antennal activity sensitivity to various release rates and proportions of ethanol and α-pinene. Bestmann and Dippold (1983) reported changes in antennal responses of three moth species when stimulated with an identical amount of sex pheromone at various temperatures. In all studied species, the smallest antennal responses were recorded at minimum and maximum temperatures. The strongest antennal responses were recorded at all medium range temperatures for one moth species,

at the lower range of mean temperatures for a second moth species, and at the temperature close to the maximum tested for the third species. Temperature has also been reported to affect the time of day and intensity of insect reproductive flight (Cardé and Roelofs, 1973; Comeau et al., 1976; Linn et al., 1988; Bento et al., 1993), performance during upwind flight (Castrovillo and Cardé, 1979; Charlton et al., 1993; Facundo et al., 1994), or perception of odor quality in insects (Linn et al., 1991).

Ambient air temperature has not previously been considered as a factor affecting the olfactory sensitity of *T. piniperda.* Our data suggest that beetle responsiveness to varying proportions to ethanol and α-pinene may be temperature dependent. In 1995, beetles were most attracted to the lures with low and high release rates of ethanol and α-pinene at maximum daily temperatures of 10–12°C for three days and 18–21°C for another three days of reproductive flight. On March 31 and April 1, 1996, the maximum daily temperatures were 15–17°C, and beetles were most attracted to the lures with medium release rates of ethanol and α-pinene. On April 11 and 15–17, 1996, the maximum daily temperatures were 17–19°C, and beetles were most attracted to the lures with low release rates of ethanol and α-piene. In 1997, the maximum daily temperatures were 12, 16, and 18–24°C, and beetles were most attracted to the lures with low and high release rates of ethanol and α-pinene. Our data suggest that at a low range of maximum daily temperatures (10–13°C), *T. piniperda* is most attracted to the lures with α-pinene and high release rates of ethanol, at 14–17°C it is most attracted to those with medium release rates of ethanol, and at 18°C and higher it is most attracted to those with low release rates of ethanol. Our data suggest that the amount of released ethanol has a direct effect on its synergism in *T. piniperda* attraction to α-pinene since the proportions between their release rates across measured temperatures remained unchanged.

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ANTIGNAWING FACTOR DERIVED FROM *Cinnamomum cassia* BARK AGAINST MICE

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Abstract—The antignawing activity of cinnamomi cortex (the dried bark of *Cinnamomum cassia*) derived materials against laboratory-reared mice was evaluated using a wire-dipping method. The biologically active component of *C. cassia* bark was characterized by spectral analysis as cinnamaldehyde. The antignawing activity of the compound was compared with that of four commercially available compounds: cinnamyl alcohol, *trans*-cinnamic acid, eugenol, and salicylaldehyde. Cinnamaldehyde and cinnamyl alcohol exhibited potent repellent activity, whereas little or no activity was produced from salicylaldehyde. Moderate activity was observed in *trans*-cinnamic acid and eugenol. Cinnamaldehyde and cinnamyl alcohol exhibited potent and moderate repellent activity at 5 and 2.5% solutions, respectively, of these compounds in ethanol. Little or no activity was observed in 1% solution. As a naturally occurring repellent, cinnamaldehyde and cinnamyl alcohol could be useful as a new preventive agent against various kinds of damage caused by rodents.

Key Words—Rodent, mouse, repellent, antignawing activity, wire-dipping method, *Cinnamomum cassia*, cinnamaldehyde, cinnamyl alcohol.

INTRODUCTION

Rodents not only cause great damage in agriculture and forestry, but also generate serious problems for communication systems, structures, and human health (Mohammad et al., 1986; Prakash, 1988a). Current control of rodent populations is primarily dependent upon continued or repeated applications of conven-

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tional rodenticides. Although many successes have been achieved with rodenticides (Mohammad et al., 1986; Prakash, 1988a), their extensive use for several decades has led to the development of resistance (MacNicoll, 1986; Hodgson et al., 1991), persistence of residues, adverse effects on nontarget organisms, environmental problems, and human health hazards (Lund, 1983; Dubock, 1986; Hayes and Laws, 1991). The decreasing efficacy of rodenticides and increasing concern about their adverse effects have necessitated development of safe and ecofriendly alternatives.

Plants may be an alternative to currently used rodent control agents because they constitute a rich source of bioactive chemicals, many of which biodegrade to nontoxic products (Swain, 1977; Harborne, 1993). Because for each rat killed with poison there are others in the area that survive, the most suitable alternative may be nonlethal chemicals such as antifertility agents or repellents (Marsh, 1988; Meehan, 1988; Nolte et al., 1993). However, relatively little work has been carried out on plant-derived repellents compared to other aspects of rodent control. Effectiveness of roden repellents might depend partly on thermal irritant by contact, olfactory avoidance, and taste aversion (Howard et al., 1968; Rogers, 1978; Meehan, 1988; Beauchamp and Mason, 1991). We have reported the repellent activity of methanol extracts from a total of 54 aromatic plant species with strong bitterness or odor (Yun et al., 1998). Among the 54 plant species, *Cinnamomum cassica* bark was highly resistant to rodent attacks.

In the laboratory study described herein, we assessed the antignawing activity of *Cinnamomum cassica* bark-derived components to laboratory-reared mice.

METHODS AND MATERIALS

Chemicals. Eugenol, *trans*-cinnamic acid, and salicylaldehyde were purchased from Sigma (St. Louis, Missouri). Cinnamyl alcohol was supplied by Tokyo Kasei (Tokyo, Japan).

Animals and Housing Conditions. Four-week-old female mice (Slc: ICR strain, 22–24 g) were purchased from Sam Yook Animal Co., Osan, Kyungi Province, Korea. Animals were allowed to acclimate to their new housing for two weeks prior to the test at approximately six weeks of age. Mice were randomly assigned to five or six groups and housed, five per plastic cage, on aspen chip bedding under conditions of controlled temperature (25 ± 2°C), 50–60% relative humidity, and a photoregime of 12L : 12D. Cages and food containers were changed every two days. Roden Chow (Sam Yook Animal Co., Ltd.) and water were provided ad libitum.

Isolation and Identification of Bark Compounds. Bark (3.6 kg) from *Cinnamomum cassica* (Family Lauraceae) was purchased as a commercially available product, dried in an oven at 60°C for two days, ground to a fine powder,

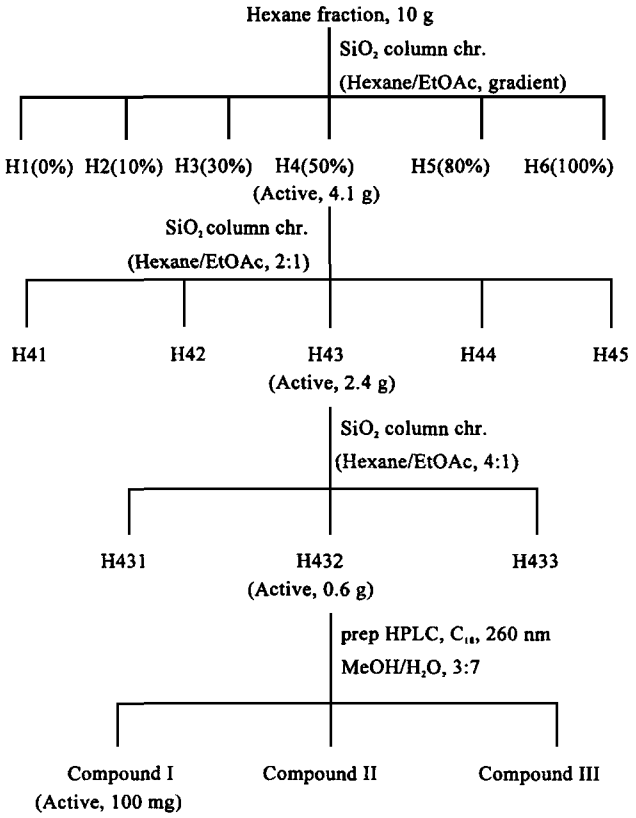

FIG. 1. Isolation procedures for antignawing compounds in methanol extracts of *Cinnamomum cassia* bark against mice.

extracted twice with methanol (10 liters) at room temperature, and filtered (Toyo filter paper No. 2, Japan). The combined filtrate was concentrated in vacuo at 35°C to yield about 10.4% (based on the weight of the bark). The extract (20 g) was sequentially partitioned into hexane (3.9 g), chloroform (4.5 g), ethyl acetate (1.9 g), and water-soluble (9.7 g) portions for subsequent bioassay with mice. The organic solvent portions were concentrated to dryness by rotary evaporation at 35°C, and the water portion was freeze-dried. For isolation, a 5% solution of each *C. cassia* bark-derived fraction in ethanol was used.

The hexane portion (10 g) was chromatographed on a silica gel column (Merck 70–230 mesh, 500 g, 5.5 cm ID × 70 cm), and successively eluted with a stepwise gradient of hexane-ethyl acetate (Figure 1). The active 50% fraction was chromatographed on a silica gel column and eluted with hexane-ethyl acetate

(2 : 1). Column fractions were collected and analyzed by TLC (hexane–ethyl acetate 3 : 1). Fractions with a similar TLC pattern were combined. The active fraction was chromatographed on a silica gel column and eluted with hexane–ethyl acetate (4 : 1). For further separation of the biologically active substance(s), a Waters Delta Prep 4000 HPLC was used. The column was 2.9 cm ID × 300 mm Bondapak C_{18} (Waters) using methanol–water (3 : 7) at a flow rate of 10 ml/min and detection of 260 nm. Finally, a potent active principle (100 mg) was isolated.

Structural determination of the active isolate was based on spectral analysis. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM-500 spectrometer, and chemical shifts are given in parts per million. UV spectra were obtained on a Waters 490 spectrometer, IR spectra on a Biorad FT-80 spectrophotometer, and mass spectra on a Jeol JMS-DX30 spectrometer.

Bioassay. The antignawing activity of the *Cinnamomum*-derived materials against mice was examined by use of the wire-dipping method (Ahn et al., 1995). The most important factor in the screening for repellent activity against rodents may be the starting concentration. A concentration of 5–10% ethanol solution (dried plant extract–ethanol, w/v) did not cause any problem with solubility and allowed detection of minor active compounds (Ahn et al., 1995). Plastic-coated flexible electric cable was cut into 10-cm segments. The segments were dipped in the 5% ethanol solution of the *Cinnamomum*-derived materials for 3 min and allowed to dry. Control segments were prepared by dipping the cable in ethanol for 3 min. Ten segments treated with each test material, 10 control segments, and five female mice were placed in the same cage under the same conditions mentioned above. All tests were replicated three times. The mouse-induced damage of the segments was observed at three days after treatment for a rapid isolation of active components.

The persistence of the repellent effects of the active isolate (cinnamaldehyde) and four commercially available *Cinnamomum* bark-derived compounds (cinnamyl alcohol, *trans*-cinnamic acid, eugenol and salicylaldehyde) (Namba, 1986; Buckingham, 1992) on mice was measured by the method mentioned above, with 5% ethanol solutions of the test materials at three-day intervals up to a 15-day period. All tests were replicated three times.

The antignawing activity of the *Cinnamomum*-derived materials was determined by weight lost due to gnawing in each wire segment treated with test materials and controls.

Statistical Analysis. Data were subjected to a one-way ANOVA. Means were compared and separated by Scheffe's test at the $P = 0.05$ level (SAS, 1995).

RESULTS

During the initial experiments, we observed that the methanol extract of *C. cassia* bark possessed repellent activity on mice. Five percent ethanol solu-

TABLE 1. ANTIGNAWING ACTIVITIES OF SOLVENT FRACTIONS OF *Cinnamomun cassia* BARKS, WIRE-DIPPING METHOD, 72 HOUR[a]

Solvent fraction[b]	Weight gnawed (mg)[c]
Hexane	2.3 ± 0.2c
Chloroform	9.1 ± 0.2a
Ethyl acetate	4.3 ± 0.4b
Butanol	10.1 ± 0.1a
Water	9.6 ± 0.4a

[a]Each data point represents the mean (±SE) of three replicates.
[b]Five percent ethanol solution.
[c]Mean followed by the same letter in each column are not significantly different [$P = 0.05$; Scheffe's test (SAS, 1995)].

tions of hexane and ethyl acetate fractions from the extract showed strong and moderate antignawing activity against mice (Table 1). Little or no activity was produced from chloroform, butanol, and water fractions. One isolate showed antignawing activity, and it was characterized by spectral analysis as *trans*-cinnamaldehyde (100 mg). The compound was identified based on the following evidence: C_9H_8O (MW, 132); EI-MS (70 eV) m/z (% rel. int.): M+ 132 (3), 103 (2), 74 (83), 59 (100), 58 (75); IR (neat) max cm^{-1}: 2920, 1680, 1630, 1130; ^1H NMR (CD_3OD, 400 MHz); 6.60 (dd, J = 8 and 18 Hz), 7.35 (d, J = 18 Hz),

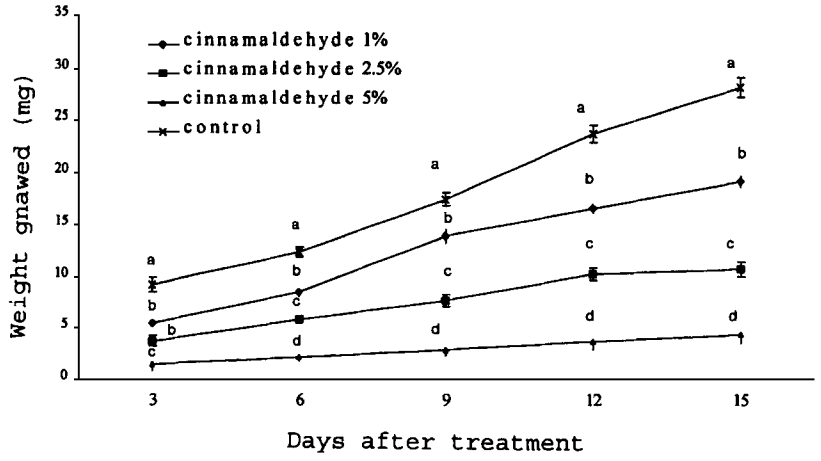

FIG. 2. Antignawing activities of *trans*-cinnamaldehyde during 15-day test determined by wire-dipping method.

TABLE 2. ANTIGNAWING ACTIVITIES OF TEST MATERIALS DURING 15-DAY TEST DETERMINED BY WIRE-DIPPING METHOD[a]

Test material[b]	Antignawing activity (mg)[c]				
	Day 3	Day 6	Day 9	Day 12	Day 15
trans-Cinnamaldehyde	2.0 ± 0.5c	2.6 ± 0.6c	3.4 ± 0.6c	4.2 ± 0.4e	5.1 ± 0.2d
Cinnamyl alcohol	1.8 ± 0.4c	3.1 ± 0.2cd	4.2 ± 0.2c	6.5 ± 0.5de	7.1 ± 0.2d
Salicylaldehyde	6.2 ± 0.5ab	10.2 ± 0.7ab	15.2 ± 0.7a	18.6 ± 0.7b	20.4 ± 0.5b
Eugenol	2.9 ± 0.5c	6.5 ± 0.7bc	8.3 ± 0.6b	10.5 ± 0.6c	11.2 ± 0.4c
trans-Cinnamic acid	3.2 ± 0.5bc	6.3 ± 1.1cd	8.3 ± 0.5b	9.9 ± 0.4cd	11.5 ± 0.3c
Untreated	8.7 ± 0.8a	11.4 ± 0.5a	16.1 ± 0.9a	25.5 ± 1.1a	27.9 ± 0.7a

[a]Each data point represents the mean (±SE) of three replicates.
[b]Five percent ethanol solution.
[c]Means followed by the same letter in each column are not significantly different [$P = 0.05$; Scheffe's test (SAS, 1995)].

7.1–7.7 (m), 9.52 (d, J = 8 Hz); ^{13}C NMR (CD_3OD, 100 MHz): 195.6, 154.4, 135.0, 132.1, 129.7, 129.7, 129.5, 129.0, 128.9.

The persistence of the repellent effects of the test materials on mice was determined over a 15-day period (Table 2). There was a significant difference in antignawing activity among the treatments ($P = 0.05$). Untreated wire segments lost more weight during the test period. Cinnamaldehyde and cinnamyl alcohol showed potent antignawing activity for approximately 15 days, whereas little or no activity was observed for salicylaldehyde during the test period. Cinnamic acid and eugenol revealed moderate antignawing activity.

Cinnamaldehyde was diluted to provide lower concentrations for titration studies because of its potent antignawing activity (Figure 2). A 5% solution of this compound in ethanol revealed significant antignawing activity against mice, whereas little or no activity was produced from 1% solution. Moderate antignawing activity was obtained in 2.5% solution of this compound.

DISCUSSION

Rodents exhibit shyness behavior after exposure to toxic chemicals (Prakash, 1988b). Prakash (1976) reported that the shyness among rodents persists for 35 to 150 days. The adverse effects and shyness of poisons calls for alternative control agents such as repellents rather than attempts to kill the rodents.

Certain plant-derived extracts have been found to have repellent properties against rodents (Harding, 1985; Hansson, 1988; Yun et al., 1998). Jacobson (1989) pointed out that the most promising botanical repellents are in the plant families Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labiatae, and Canellaceae. Plant-derived repellent properties towards rodents were also reported in

Thujopsis dolabrata var. *hondai* (Cupressaceae) (Ahn et al., 1995), *Artemsia absinthium* (Compositae) (Harding, 1985), and *Aucklandia lappa* (Compositae), *Illicium verum* (Magnoliaeceae), *Rheum officinale* (Polygonaceae), and *Pinus densiflora* (Pinaceae) (Yun et al., 1998). In our study, methanol extract from *C. cassia* (Family Lauraceae) bark exhibited potent antignawing activity. This plant species is important not only as a spice, but in East Asia is considered to have some medicinal properties, such as a stomach agent, an astringent, and a carminative agent (Namba, 1986).

Various compounds, including phenolics, terpenoids, and alkaloids exist in plants and jointly or independently are repellent to animals (Harding, 1985; Hansson, 1988; Harborne, 1993). They act in many ways on various rodent species and may be applied to the cables, structures, or agricultural products in the same way as other agricultural chemicals. Many *Cinnamomum* species are rich in essential oil and tannins (Morimoto et al., 1986; Namba, 1986; Buckingham, 1992). In our study, the responses varied with the chemical tested. Cinnamaldehyde and cinnamyl alcohol were the most potent of the five components examined. These compounds might form a new source for managing rodents. Recent investigations have shown that cinnamaldehyde has antifungal (Thompson, 1989), antibacterial (Lee and Ahn, 1998), and antimutagenic effects (Kakinuma et al., 1984).

Some plant-derived extracts and phytochemicals were effective against mice for only a relatively short period, typically a few days (Harding, 1985; Ahn et al., 1995). For example, carvacrol was effective against mice for a short period, probably due to its high volatility in air. However, the rodent repellent cycloheximide from *Streptomyces griseus* is effective for more than one month as a 5% solution in ethanol (Ahn et al., 1995). It has been reported that effectiveness of rodent repellents might depend partly on a thermal irritant by contact, olfactory avoidance, and taste aversion (Howard et al., 1968; Rogers, 1978; Meehan, 1988; Beauchamp and Mason, 1991). Effectiveness of carvacrol and cycloheximide might depend partly on these same properties (Ahn et al., 1995). Due to the high volatility of cinnamaldehyde (Budavari et al., 1989), its effectiveness also might depend partly on olfactory avoidance, although the exact repellent mechanism remains unknown. If cinnamaldehyde is an olfactory repellent, it might be effective as a pest control agent in limited spaces such as storage bins, greenhouses, or buildings because of its high volatility (Budavari et al., 1989). An appropriate carrier will be needed for slow release into the air. Harding (1985) recommended a suitable liquid or solid carrier such as lacquer, sodium silicate, or paraffin. A combination of thujone oil and lacquer (12/88), when used like a paint, has been reported to keep rodents away from the painted area for between three and five years (Harding, 1985).

Based upon our data and these earlier findings, cinnamaldehyde would be expected to protect against damage by rodents for a relatively long time if a carrier with slow-releasing properties were developed.

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PRODUCTION OF PHENOLS AND SPINES IN RESPONSE TO SHOOT DAMAGE IN *Prosopis caldenia* and *Prosopis flexuosa*

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Abstract—Shoot damage affects the chemical and physical defenses of woody plants. We studied how branch defoliation and intensive shoot damage affected leaf phenol concentration and spinescence in *Prosopis caldenia* and *P. flexuosa*. Individuals of both species were branch defoliated, heavily pruned, or naturally burnt. At the end of the growing season, stump (from pruned plants) and burnt sprouts, defoliated branches, and branches from intact control plants were harvested for assessment of phenols and spinescence. In both species, foliar phenol content in stump or burnt sprouts and defoliated branches were similar or below that of intact control branches. The biomass invested in spines was similar, spine length lower, and spine density higher in stump or burnt sprouts than in control branches in both species. These findings are discussed in relation to the juvenile-reversion hypothesis and the carbon/nutrient balance hypothesis.

Key Words—Antiherbivore defenses, phenols, spines, defoliation, pruning, burning, *Prosopis caldenia*, *Prosopis flexuosa*, Argentina.

INTRODUCTION

Shoot damage affects the level of antiherbivore defenses of woody plants. Intensive shoot damage of mature plants (e.g., burnt, topping) causes a genetic reversion to a juvenile phase of growth (Kozlowski, 1971) and, since woody plants are at high risk of herbivory when juvenile, it has been hypothesized that the juvenile phase of woody plants is characterized by an increased allocation of carbon to chemical and/or physical defenses (Bryant et al., 1983, 1991a). A specific

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prediction from the juvenile-reversion hypothesis is that defense levels in sprouts from heavily pruned plants (stump sprouts) or burnt plants (burnt sprouts) will be higher than in intact control branches.

Less intensive shoot damage of mature plants, such as defoliation, can also increase the level of defenses in the regrowth (Karban and Myers, 1989; Tallamy and Raupp, 1991). Increases in defenses following defoliation have often been interpreted as an active response to tissue injury (Rhoades, 1979; Haukioja, 1980; Karban and Myers, 1989; Tallamy and Raupp, 1991). However, changes in plant defenses following herbivory may be the consequence of internal and/or external constraints limiting plant primary metabolism (Bryant et al., 1991b; Coleman and Jones, 1991; Tuomi et al., 1991). The carbon/nutrient (hereafter C/Nu) balance hypothesis (Bryant et al., 1983) is a current phenotypic explanation for changes in carbon-based defenses following herbivory. The C/Nu balance hypothesis states that the changes in the relative amount of carbon and nutrients after plant tissue removal determine the allocation of carbon to defenses. In deciduous woody species, defoliation (removal of leaves only) is predicted to reduce mineral nutrient reserves more rapidly than carbohydrate reserves, thereby causing a nutrient limitation of growth and an increased allocation of carbon to defenses (Bryant et al., 1991b). The C/Nu balance hypothesis was originally formulated to explain the effects of resource imbalances on whole plants. However, assuming independence of plant parts or units (Watson and Casper, 1984), the outcome of defoliation would be the same irrespective of the level of defoliation (single branch or whole tree) (Tuomi et al., 1991). Therefore, a specific prediction from the C/Nu balance hypothesis is that defense levels in the regrowth of previously defoliated branches will be higher than in intact control branches in deciduous woody plants.

In arid and semiarid habitats, chemical defenses of woody plants are mainly represented by phenols and terpenoids (Meyer and Karasov, 1991). Phenols show antiherbivore effects such as toxicity and reduction in the efficiency of nutrient utilization (Robbins et al., 1987; Lindroth, 1989; Hagerman et al., 1992; Cheeke and Palo, 1995), which result in reduced acceptability by herbivores (Provenza, 1995). On the other hand, physical defenses are represented by a variety of structural and morphological characteristics, which include hairs, prickles, spines, and thorns. Spines and thorns are common in arid habitats and are often interpreted as adaptations to protect plants from large herbivores (Myers and Bazely, 1991). Spinescence has been shown to reduce feeding rate of mammalian herbivores (Cooper and Owen-Smith, 1986; Belovsky et al., 1991). Moreover, recent results suggest that spine length may protect leaves (Milewski et al., 1991), whereas spine density may protect twigs against mammalian herbivore (Gowda, 1996).

Prosopis caldenia and *P. flexuosa* are native, deciduous woody plants in central Argentina. Both species are high in leaf total phenols and possess a pair of straight long spines at each axillary bud (Pisani and Distel, 1998). There is no information on how branch defoliation or severe shoot damage affects the

level of defenses in these species. The objective of this study was to assess for both species (1) the level of leaf phenols in the regrowth of previously defoliated (manually) branches, (2) the level of leaf phenol and spinescence (biomass invested in spines, spine density, and spine length) in sprouts from heavily pruned plants (stump sprouts) and in sprouts from naturally burnt plants (burnt sprouts), and (3) the level of leaf phenols and spinescence in intact control branches. In addition, the foliar biomass in defoliated and intact branches and the current season twig production of intact branches were measured.

METHODS AND MATERIALS

Study Site and Species. The study was conducted in the Caldén District (Cabrera, 1976), on a site located in the southeastern zone of La Pampa province (38°45′S; 63°45′W), Argentina. The climate is temperate, semiarid. Mean monthly temperatures range from 7°C in July to 24°C in January, with an annual mean of 15°C. Mean annual rainfall is 400 mm, with peaks in March and October. The more severe droughts occur in summer. Precipitation in the study period was 393, 396, 363, and 267 mm, in 1992, 1993, 1994, and 1995, respectively. Dominants soils are classified as Calciustolls. In uplands, they present a petrocalcic horizon at 60–80 cm depth. The physiognomy of the vegetation is shrubland. Woody species are mainly represented by *P. caldenia*, *P. flexuosa*, *Larrea divaricata*, and *Condalia microphilla*, whereas herbaceous species are represented by several species in the genus *Stipa*, *Piptochaetium napostaense*, *Poa ligularis*, *Medicago minima*, and *Erodium cicutarium.*

P. caldenia and *P. flexuosa* are warm-season, leguminous trees, native to Argentina (Burkart, 1976). They normally start producing leaves in October and lose their leaves in April (Distel and Peláez, 1985). Presently both species are subjected to low herbivory from wild and domestic animals, except for the pods, which are heavily eaten. Defensive traits such as phenols and spines might have resulted from evolutionary interaction with megafauna during the Pleistocene (Janzen and Martin, 1981; Bucher, 1987).

Field Experiment. Sixty adult plants each of *P. caldenia* and *P. flexuosa* were randomly selected in an upland site in the spring of 1992. Plants were the same size (2–3 m high) and were growing on similar soil. They were at the mature stage of growth (sensu Kozlowski, 1971), as evidenced by a complex branch structure and the production of pods (fruits). On December 15 (1992), 20 plants of each species were cut at 25 cm above soil surface. This treatment simulated disturbance from intensive fires, which are common in the region (Bóo, 1990). In another 20 plants of each species, four branches of the same basal diameter (8 mm) and ramification order were defoliated on each tree. The remaining 20 plants were untreated and served as controls. In the first week of April

(end of growing season) 1993, stump sprouts, defoliated branches, and similar branches randomly selected from control plants were harvested. The same procedure was repeated in the 1994–1995 growing cycle, except that the number of plants per treatment was reduced to 10. Different plants were used in the 1994–1995 growing cycle.

Samples were sorted into leaves and stems, air-dried, and weighed. A low drying temperature (20–30°C) minimizes denaturing of carbon-based secondary metabolites (Hagerman, 1988). Leaves were ground to pass through a 1-mm sieve and analyzed for total phenols following the method of Price and Butler (1977). Total phenols were extracted from 60 mg of leaf powder with 3 ml of methanol for 15 hr at room temperature. The extract was filtered, the extraction tube rinsed with an additional 3 ml of methanol, and the filtrate added to 50 ml of distilled water. Then, 3 ml of 0.1 M $FeCl_3$ in 0.1 N HCl was added to the extract. Exactly 20 min after the addition of the ferric chloride, 3 ml of 0.008 M $K_3Fe(CN)_6$ was added. Finally, 20 min after the addition of the ferricyanide, an aliquot of 1.5 ml of methanolic extract was diluted by the addition of 1.5 ml of distilled water, and the absorbance read in a 1-cm glass cuvette at 720 nm. A blank of identical composition, but omitting the leaf extract, was analyzed and subtracted from all other readings. Phenol concentrations were expressed as absorbances, as recommended by Martin and Martin (1982).

Untreated branches and stump sprouts were used to determine biomass invested in spine (milligrams of spine per gram of branch), spine density (number spines per centimeter of branch), and spine length (millimeters). Untreated branches were also used to estimate the production of current season twigs.

Field Observation. In the third week of February 1993, a natural fire occurred in a lowland site, located at 2 km from the upland site where the field research was conducted. Fire intensity was enough to destroy the foliage and small branches, but not enough to damage growth buds on large branches. Consequently, these branches produced sprouts. Seven weeks later, 20 plants of *P. caldenia* and 20 plants of *P. flexuosa* were selected in the burnt site. A similar number of plants of each species was selected in an adjacent unburnt site, and served as control. Four sprouts (burnt site) or four branches (unburnt site) of 8 mm in diameter were harvested from each of the selected plants. Samples were analyzed for total phenols and spinescence following the procedures described in the Field Experiment section above.

Statistical Analysis. The data were analyzed by t tests, escept for foliar phenols in stump sprouts, defoliated branches, and intact branches. The latter data were analyzed by one-way ANOVA. The biomass invested in spines and the length of spines were ln-transformed prior to analysis. Nontransformed variables are shown in the tables below. The data on production of current season twigs and spine density were analyzed by the Kruskal-Wallis nonparametric test. A difference between means was considered significant at $P < 0.05$.

TABLE 1. FOLIAR BIOMASS IN DEFOLIATED AND CONTROL INTACT BRANCHES AT END OF GROWING SEASON

	Biomass (g/branch)[a]	
	P. caldenia	*P. flexuosa*
1993		
Defoliated	1.5 ± 0.2	0.8 ± 0.2
Control	2.3 ± 0.2	4.1 ± 0.5
P	0.003	<0.001
1995		
Defoliated	3.0 ± 0.3	3.9 ± 0.9
Control	3.4 ± 0.4	4.6 ± 0.5
P	0.409	0.105

[a]Foliar biomass produced after defoliation. Values are means ±1 SE (N = 14–20, 1993; N = 9–10, 1995). P values are from t tests.

RESULTS

In both specie, mean foliar biomass of defoliated branches was below that of controls in 1993, but similar to controls in 1995 (Table 1). Current season twig biomass was higher in 1995 than in 1993 (Table 2).

Branch defoliation resulted in foliar phenol content higher than (*P. caldenia*) or similar to (*P. flexuosa*) controls in 1993, but below that of controls for both species in 1995 (Table 3). Foliar phenol content in stump sprouts of *P. caldenia* was higher than (1993) or similar to (1995) controls, whereas in *P. flexuosa* was below that of the control in both years (Table 3). The content of phenols in leaves of the sprouts from burnt plants was lower than that of unburnt controls (Table 4).

In both species, the biomass of spines was similar, spine length lower, and spine density higher in stump or burnt sprouts than in control branches (Table 5).

TABLE 2. CURRENT SEASON TWIG BIOMASS IN CONTROL INTACT BRANCHES

	Biomass (g/branch)[a]	
	P. caldenia	*P. flexuosa*
1993	0.2 ± 0.1	0.9 ± 0.4
1995	2.4 ± 0.4	2.6 ± 0.7
P	<0.001	0.008

[a]Values are means ± 1SE (N = 20, 1993; N = 10, 1995). P values are from Kruskal-Wallis nonparametric test.

TABLE 3. TOTAL FOLIAR PHENOLS IN SPROUTS FROM CUT PLANTS, DEFOLIATED BRANCHES, AND CONTROL INTACT BRANCHES[a]

	A_{720}/60 mg dry weight	
	P. caldenia	*P. flexuosa*
1993		
Stump sprout	1.48 ± 0.03 a	1.23 ± 0.04 b
Defoliated	1.45 ± 0.03 a	1.45 ± 0.04 a
Control	1.26 ± 0.03 b	1.39 ± 0.02 a
1995		
Stump sprout	1.18 ± 0.07 b	0.88 ± 0.05 a
Defoliated	1.05 ± 0.02 a	1.10 ± 0.04 b
Control	1.21 ± 0.08 b	1.44 ± 0.09 c

[a]Values are means ± 1SE (N = 14–20, 1993; N = 8–10, 1995). Different letters in each species and year indicate significant ($P < 0.05$) differences [LSD test (Snedecor and Cochran, 1980)].

DISCUSSION

Assuming independence of branches, the C/Nu balance hypothesis predicts that in deciduous woody plants defense levels in the regrowth of previously defoliated branches will be higher than in intact control branches. However, we found that the concentration of foliar phenol in defoliated branches was either above (1993) or below (1995) that of the control intact branches. Moreover, there was a negative relationship between growth and the concentration of foliar phenols in defoliated branches of *P. caldenia* and *P. flexuosa.* A strong negative correlation between growth and production of secondary metabolites has been shown for a number of species (e.g., Gershenzon, 1984; Herms and Mattson, 1992). The C/Nu hypothesis may fail to predict chemical changes at the branch defoliation level because it does not take in account changes in branch growth activity after defoliation (Honkanen and Haukioja, 1994). Our results showed that when

TABLE 4. TOTAL FOLIAR PHENOLS IN SPROUT OF BURNT PLANTS AND IN CONTROL INTACT BRANCHES[a]

	A_{720}/60 mg dry weight	
	P. caldenia	*P. flexuosa*
Burnt sprout	1.18 ± 0.02	1.18 ± 0.02
Control	1.39 ± 0.03	1.41 ± 0.02
P	<0.001	<0.001

[a]Values are means ± 1SE (N = 19–20). P values are from t tests.

TABLE 5. SPINESCENCE IN STUMP AND BURNT SPROUTS AND INTACT CONTROL BRANCHES[a]

	Biomass invested in spines (mg/g branch)		Spines density (*N*/cm branch)		Spines length (mm)	
	P. caldenia	*P. flexuosa*	*P. caldenia*	*P. flexuosa*	*P. caldenia*	*P. flexuosa*
1993						
Stump sprout	27.2 ± 5.7	17.6 ± 3.4	1.17 ± 0.08	0.76 ± 0.08	7.8 ± 0.41	5.9 ± 0.65
Control	39.1 ± 6.2	32.7 ± 7.2	0.64 ± 0.03	0.36 ± 0.02	11.5 ± 0.97	10.2 ± 0.88
P	0.325	0.259	<0.001	<0.001	0.002	<0.001
1995						
Stump sprout	35.8 ± 7.02	33.8 ± 9.06	1.34 ± 0.09	1.01 ± 0.07	9.3 ± 0.96	7.2 ± 0.31
Control	42.3 ± 14.60	30.8 ± 6.85	0.71 ± 0.07	0.54 ± 0.04	14.5 ± 1.67	11.8 ± 1.11
P	0.613	0.818	0.001	0.001	0.020	0.002
1993						
Burnt sprout	25.3 ± 4.15	24.0 ± 3.30	1.13 ± 0.04	0.77 ± 0.04	6.1 ± 0.29	6.0 ± 0.34
Control	33.6 ± 4.26	31.1 ± 8.10	0.62 ± 0.03	0.38 ± 0.04	11.1 ± 0.83	8.5 ± 1.03
P	0.166	0.511	<0.001	<0.001	<0.001	0.029

[a]Values are means ± 1SE (N = 18–20, 1993; N = 8–10, 1995). P values are from t tests for biomass invested in spines and spine length, and from Kruskal-Wallis nonparametric test for spine density.

conditions for growth were relatively favorable (1995), defoliated branches were able to compensate for the lost foliage and leaf phenol concentration was below that of intact control branches. Conversely, when conditions for growth were relatively unfavorable (1993), defoliated branches were unable to compensate for the loss of foliage and leaf phenol concentration was above that of intact control branches.

On the other hand, the juvenile-reversion hypothesis predicts that defense levels in sprouts from heavily pruned plants (stump sprouts) or burnt plants (burnt sprouts) will be higher than in intact control branches. We found that both types of sprouts were similar or lower in leaf phenols (except for *P. caldenia* in 1993) and spinescence than intact control plants, with the exception of spine density. Spine density was consistently higher in stump and burnt sprouts than in intact control branches, which supports the prediction from the juvenile-reversion hypothesis. Shoot internodes were shorter (P = 0.001) in stump and burnt sprouts (1.58 cm ± 0.05 SE) than in control branches (2.19 cm ± 0.10 SE) in both species. Moreover, the proportion of axillary buds lacking spines was lower (P = 0.002) in stump and burnt sprouts (0.23 ± 0.01 SE) than in intact control branches (0.33 ± 0.04 SE). This pattern of internode and spine growth resulted in a higher density of spines in sprouts than in control branches from adult plants. Moreover, a high density of spines was also observed in seedlings of both species (Pisani and Distel, 1998). Increases in spine density has been shown to be effective in protecting stems against herbivores (Gowda, 1996).

Concordantly, goats removed their food by picking (removal of leaves only) when feeding on stump sprouts from both species under study (J. M. Pisani and R. A. Distel, unpublished data). Taken together, the results on leaf phenols and spine production suggest that during the juvenile phase of growth of *P. caldenia* and *P. flexuosa*, protection of stems may be more important than protection of leaves in terms of loss of fitness if eaten by herbivores.

In conclusion, our results suggest that hypotheses on the effects of defoliation in the production of carbon-based defenses in regrowth should take into account activity after defoliation. Furthermore, some "induced" defenses (spine density in this study) associated with juvenile-phase reversion may simply result from alterations in plant structure.

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RESOLUTION OF EPOXYDIENES BY REVERSED-PHASE CHIRAL HPLC AND ITS APPLICATION TO STEREOCHEMISTRY ASSIGNMENT OF MULBERRY LOOPER SEX PHEROMONE

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Abstract—Resolution of insect pheromonal *cis*-epoxydiene racemates derived from (*Z*,*Z*,*Z*)-3,6,9-trienes was examined with a reversed-phase chiral HPLC column. The results showed that a Chiralcel OJ-R column was suitable for separating the enantiomers having a C_{17}–C_{23} unsaturated straight chain except for 9,10-epoxydienes with a C_{21}–C_{23} chain. To determine the absolute configuration of the separated enantiomers, each of the optically active epoxydienes was hydrogenated over Pd-$BaSO_4$ and its behavior was examined on this chiral column by cochromatography with the corresponding chiral epoxy compound having a saturated chain, which was prepared via a Sharpless epoxidation reaction. This analysis showed that the dextrorotatory C_{17}–C_{23} 3,4- and 6,7-epoxydienes and C_{17}–C_{20} 9,10-epoxydienes with shorter R_ts possess (3*S*,4*R*)-, (6*S*,7*R*)-, and (9*R*,10*S*) configurations, respectively, and the levorotatory enantiomers with longer R_ts possess the opposite configuration. An abdominal tip extract of the mulberry looper, *Hemerophila artilineata* Butler (Lepidoptera: Geometridae: Ennominae), included (9*S*,10*R*)-(*Z*,*Z*)-*cis*-9,10-epoxy-3,6-octadecadiene as a main sex pheromone component. The synthetic (9*S*,10*R*)-9,10-epoxydiene, rather than its antipode, elicited strong antennal and behavioral responses from the male moths in electrophysiological and field tests.

Key Words—Chiral column, reversed-phase HPLC, resolution, sex pheromone, lepidopteran attractant, stereochemistry, epoxydiene, mulberry looper.

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INTRODUCTION

(*Z*,*Z*,*Z*)-3,6,9-Trienes with a C_{17}–C_{23} straight chain and their monoepoxy derivatives constitute one of the main groups of lepidopterous sex pheromones and attractants, particularly in families of Geometridae, Arctiidae, and Noctuidae (Arn et al., 1992, 1997; Ando et al., 1995). The *cis*-epoxydienes are chiral compounds, and several optically active pheromonal epoxides have been synthesized by using Sharpless asymmetric epoxidation of an allylic alcohol (Cossé et al., 1992; Millar et al., 1990; Millar and Underhill, 1986; Mori and Brevet, 1991; Mori and Ebata, 1986; Szöcs et al., 1993; Wong et al., 1985) or pig pancreatic lipase-catalyzed asymmetric hydrolysis of a *meso*-diacetoxyepoxybutane (Brevet and Mori, 1992). Although these chiral syntheses produced valuable authentic samples for understanding the stereochemistry of natural pheromones, many laborious steps and great skill are required to yield a final product with high enantiomeric excess (ee).

Recently, we reported the resolution of *cis*-epoxydienes by HPLC equipped with normal-phase chiral columns (Qin et al., 1997). A Chiralpak AS column was suitable for separating enantiomers from racemic 3,4-epoxydienes, and a Chiralpak AD column was indispensable for separation of racemic 6,7-epoxydienes and 9,10-epoxydienes. With this chiral HPLC, many kinds of optically pure samples can be systematically prepared without long-term efforts from the racemic epoxydienes that are easily synthesized from linolenic acid. This chiral HPLC column also is utilized for the analysis of a natural pheromone component, and we successfully determined the stereochemistry of a pheromonal 3,4-epoxydiene secreted by the Japanese giant looper (Ando et al., 1997). The separating capability of the column was, however, easily decreased after an injection of a highly concentrated sample. Because of the very low polarity of the epoxydienes, *n*-hexane with a limited amount of 2-propanol (<0.1%) was used as the solvent for their resolution, and the reproducibility of the chromatography was not sufficient. This paper deals with the resolution ability of another new chiral HPLC column operated under a reversed-phase condition, which is expected to produce reliable chromatograms of chiral epoxydienes. We also report here the stereochemistry of a main pheromone component of the mulberry looper. The larva is a harmful defoliator of mulberry, and the sex pheromone has been identified as a mixture of (*Z*,*Z*)-*cis*-9,10-epoxy-3,6-octadecadiene (*Z*3,*Z*6,epo9–18 : H,[2] main component) and (*Z*)-*cis*-9,10-epoxy-6-octadecene (Z6,epo9–18 : H, minor component) by Tan et al. (1996). They have suggested the stereochemistry from field tests of synthetic enantiomers with moderate optical activity. We examined the pheromone extract with the reversed-phase chiral HPLC column and further evaluated the biological activities of both enantiomers in electrophy-

[2]Other epoxydienes are also abbreviated in a similar manner.

siological and field tests after they were completely resolved from racemic *Z*3,*Z*6,epo9–18 : H.

METHODS AND MATERIALS

Chiral Column Chromatography. The HPLC used was a Jasco PU-980 liquid chromatograph equipped with an integrator (System Instrument Chromatocorder 21J), RI detector (Labo System RI-98Scope), and UV spectrometric detector (Jasco UV-970) operated at 215 nm. Epoxydienes were injected into a Chiralcel OJ-R column (0.46 cm ID × 15 cm, Daicel Chemical Industry Co., Ltd, Tokyo, Japan) by using 10 or 15% water in methanol as solvents at a flow rate of 0.5 ml/min. In addition, the natural pheromone of the mulberry looper was also analyzed on a Chiralpak AD column (0.46 cm ID × 25 cm, Daicel Chemical Industry Co.). For this normal-phase chiral HPLC, 0.075% 2-propanol in *n*-hexane was used as the solvent at a flow rate of 0.45 ml/min.

Unsaturated Epoxides. Synthesis, purification, and chemical characterization of epoxydienes with a C_{17}–C_{23} chain and an epoxymonoene with a C_{18} chain have been reported in detail (Ando et al., 1993, 1995).

Saturated Epoxides. cis-3,4-Epoxyicosane ($[\alpha]_D$ = +0.4°) with predominantly (3*S*,4*R*) configuration was synthesized according to the procedure for (6*S*,7*R*)-*Z*3,epo6,*Z*9–19 : H reported by Mori and Brevet (1991). HPLC analysis on an OJ-R column with 10% water in methanol as the solvent showed that this synthesis produced (3*S*,4*R*)-epoxyicosane [retention time (R_t): 19.21 min] and the (3*R*,4*S*)-isomer (R_t: 20.73 min) at a ratio of 91 : 9 (82% ee). Employing analogous methods, we synthesized mixtures of (6*S*,7*R*)-6,7-epoxyicosane (R_t: 17.35 min) and (6*R*,7*S*)-isomer (R_t: 19.36 min) in a ratio of 93 : 7 (86% ee, $[\alpha]_D$ = +0.2°), and (9*S*,10*R*)-9,10-epoxyicosane (R_t: 17.19 min) and (9*R*,10*S*)-isomer (R_t: 18.23 min) in a ratio of 90 : 10 (80% ee, $[\alpha]_D$ = +0.2°). Specific rotations of optically active epoxides in $CHCl_3$ were measured on a JASCO DIP-4 polarimeter.

Hydrogenation of Optically Active Epoxydienes. The racemate of epo3,*Z*6, *Z*9–20 : H was resolved by an OJ-R column. Fraction I (500 μg) with a shorter R_t was dissolved in *n*-hexane (1 ml) including 5% Pd-$BaSO_4$ (500 μg) and vigorously stirred under H_2 gas for 10 min. After filtration of the catalyst and evaporation of the solvent, the hydrogenated product (*cis*-3,4-epoxyicosane) was analyzed by HPLC with the chiral OJ-R column in order to compare its R_t with the corresponding compound stereoselectively prepared as described above. Fraction II of epo3,*Z*6,*Z*9–20 : H with a longer R_t and both fractions resolved from racemic *Z*3,epo6,*Z*9–20 : H and *Z*3,*Z*6,epo9–20 : H were hydrogenated in the same manner, and their stereochemistry was determined by co-chromatography with authentic samples.

Insect Culture and Pheromone Extraction. Larvae of the mulberry looper, *Hemerophila artilineata* Butler (Geometridae: Ennominae), were reared on mulberry leaves at 23°C with a 16L : 8D photoperiod. Pheromone glands were removed from moths (2 or 3 days old) in the scotophase and soaked in *n*-hexane for 30 min to extract the sex pheromone. To examine the stereochemistry of the natural pheromone, the extract was partially purified by HPLC with a Nucleosil NO_2 column (0.8 cm ID × 15 cm, packed by Senshukagaku, Tokyo, Japan) with 0.5% THF in *n*-hexane (flow rate: 1.0 ml/min) as the solvent to obtain the fraction including *Z*3,*Z*6,epo9–18 : H (R_t: 12.6 min), which then was injected to the reversed-phase chiral column. The extract was also analyzed by normal-phase chiral HPLC after purification of *Z*3,*Z*6,epo9–18 : H (R_t: 19.8 min) by a ODS column (0.8 cm ID × 15 cm, Senshukagaku, Tokyo, Japan) with 10% water in methanol (flow rate: 1.5 ml/min).

EAG Tests. This electrophysiological assay was carried out by modifying the method as described in the review by Roelofs (1984). Each test material was applied to a small piece of paper (1 × 2 cm) that was fixed in a glass syringe (5 ml). After removing the head of the male moth and placing it on tissue paper wetted with insect saline, 1 ml of air containing vapor of the chemical was puffed on one of two antennae connected to a recording electrode. The responses were recorded with a handy recorder (Pen Oscillograph, Type 8K31, NEC San-ei Instruments, Ltd., Tokyo, Japan) instead of a cathode ray oscilloscope.

Field Tests. Each enantiomerically enriched sample of *Z*3,*Z*6,epo9–18 : H (>95% ee, 300 μg) was applied to a rubber septum (8 mm OD, Aldrich Chemical Co.) in addition to racemic *Z*6,epo9–18 : H (30 μg). The lure was placed in a trap (30 × 27 cm bottom plate with a roof, Takeda Chemical Ind., Ltd., Osaka, Japan) that was set at a 1.2 m height in mulberry fields in Kanagawa Prefecture in Japan (Tsukui Farm of Tokyo University of Agriculture and Technology, Tsukui-gun) and in Su Zhou in China (Su Zhou University). Some combinations of the enantiomers (8 : 2, 5 : 5, and 2 : 8; 300 μg/septum) mixed with racemic *Z*6,epo9–18 : H (30 μg) were tested at the same time.

RESULTS

Resolution of Epoxydiene Racemates by Reversed Phase Chiral HPLC. The Chiralcel OJ-R column was suitable to separate the enantiomers of every monoepoxide derived from (*Z*, *Z*, *Z*)-3,6,9-trienes with a C_{17}–C_{23} straight chain except for C_{21}–C_{23} 9,10-epoxydienes. Table 1 shows the R_ts of both enantiomers and their separation factor (α). Chromatograms of three positional isomers with the same chain length (C_{20}) are shown in Figure 1[I] and those of three 6,7-epoxydienes with different chain length (C_{17}, C_{18}, and C_{19}) in Figure 1[II]. Since compounds with a longer chain need a longer duration for their elution on this chiral

TABLE 1. ENANTIOMERIC SEPARATION OF *cis*-EPOXYDIENES DERIVED FROM (*Z,Z,Z*)-3,6,9-TRIENES WITH A C_{17}–C_{23} CHAIN ON REVERSED-PHASE CHIRAL HPLC COLUMN[a]

	3,4-Epoxide			6,7-Epoxide			9,10-Epoxide		
	Retention time (min)			Retention time (min)			Retention time (min)		
Compound	Fraction I (+)-(3*S*,4*R*) isomer	Fraction II (−)-(3*R*,4*S*) isomer	Separation factor (α)	Fraction I (+)-(6*S*,7*R*) isomer	Fraction II (−)-(6*R*,7*S*) isomer	Separation factor (α)	Fraction I (+)-(9*R*,10*S*) isomer	Fraction II (−)-(9*S*,10*R*) isomer	Separation factor (α)
C_{17}[b]	17.59	18.90	1.09	17.03	19.54	1.19	19.09	20.33	1.08
C_{18}[b]	22.18	23.90	1.09	21.43	25.76	1.25	23.46	24.76	1.07
C_{19}[b]	27.09	29.37	1.10	26.88	33.17	1.26	28.89	29.96	1.04
C_{20}[c]	15.41	16.34	1.08	14.44	16.99	1.24	15.73	16.09	1.03
C_{21}[c]	18.06	19.06	1.07	17.07	19.84	1.20	18.19		1.00
C_{22}[c]	20.78	21.91	1.07	19.62	22.73	1.19	21.30		1.00
C_{23}[c]	23.59	24.94	1.07	22.38	25.55	1.17	24.31		1.00

[a]Chiralcel OJ-R, 0.46 cm ID × 15 cm.
[b]Solvent: 15% water in methanol, flow rate: 0.5 ml/min.
[c]Solvent: 10% water in methanol, flow rate: 0.5 ml/min.

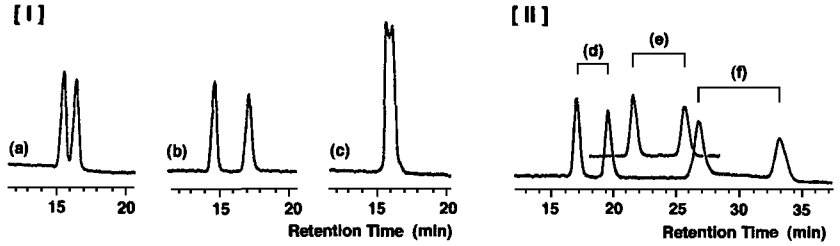

FIG. 1. Chromatograms of racemic *cis*-epoxydienes (50 μg each) derived from (*Z*,Z,Z)-3,6,9-trienes on a Chiralcel OJ-R column analyzed by an RI detector with 10% water [I] and 15% water [II] in methanol as solvents (flow rate: 0.5 ml/min). Separated compounds: (a) epo3,*Z*6,*Z*9–20 : H, (b) *Z*3,epo6,*Z*9–20 : H, (c) *Z*3,*Z*6,epo9–20 : H, (d) *Z*3,epo6,*Z*9–17 : H, (e) *Z*3,epo6,*Z*9–18 : H, and (f) *Z*3,epo6,*Z*9–19 : H.

column, similar to that on an ordinary achiral ODS column, methanol with different amounts of water was used as the solvent, i.e., 15% water for C_{17}–C_{19} epoxydienes and 10% water for C_{20}–C_{23} epoxydienes. Each enantiomer with a shorter R_t, which was recovered from the HPLC as fraction I, showed a positive value of $[\alpha]_D$, and that with a longer R_t (fraction II) showed a corresponding negative value. Enantiomers of 6,7-epoxydiene eluted at distinctively different R_ts, and their α values are larger than those of 3,4- and 9,10-dienes.

Stereochemistry of Separated Enantiomers. The absolute configuration of each separated enantiomer with a C_{20} chain was determined by analysis of its hydrogenated product on a chiral HPLC column. Fraction I of the 3,4-epoxydiene was converted into one stereoisomer of 3,4-epoxyicosane, which was coincident with a major product stereoselectively synthesized via a Sharpless epoxidation reaction utilizing (+)-tartrate, indicating that the dextrorotatory 3,4-epoxydiene with shorter R_ts possessed (3*S*,4*R*)-configuration. Fraction II was reduced to the (3*R*,4*S*) isomer, a minor product of the synthesis, thus the (3*R*,4*S*) configuration of the levorotatory 3,4-epoxydiene with longer R_ts was confirmed. By the same method, (6*S*,7*R*) and (6*R*,7*S*) configurations were assigned to the dextrorotatory fraction I and levorotatory fraction II of *Z*3,epo6,*Z*9–20 : H, respectively. Furthermore, (9*R*,10*S*) and (9*S*,10*R*) configurations were confirmed for the dextrorotatory fraction I and levorotatory fraction II of *Z*3,*Z*6,epo9–20 : H, respectively.

Chiral Column Analysis of Pheromone Extract of Mulberry Looper. To reveal the stereochemistry of a main component of the mulberry looper sex pheromone, the pheromone gland extract had to be purified before HPLC analysis on a chiral column. On examining the purification method with HPLC, it was found that a column operated in the opposite phase was serviceable, i.e., analysis with a reversed-phase chiral column after purification with a normal-phase

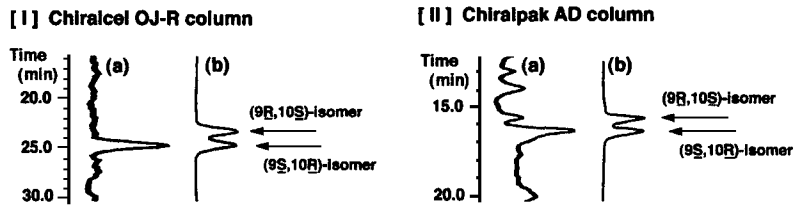

FIG. 2. HPLC chromatograms of the pheromone extract (50FE) of the mulberry looper (a) and the racemic standard of Z3,Z6,epo9–18 : H (b) on a Chiralcel OJ-R column (I) and on a Chiralpak AS column (II). Solvent systems: 15% water in methanol at 0.5 ml/min flow rate for (I), and 0.075% 2-propanol in *n*-hexane at 0.45 ml/min flow rate for (II).

achiral column, and analysis with a normal-phase chiral column after purification with a reversed-phase achiral column. Fifty female equivalents (FE) of the pheromone extract exhibited an abundant peak of the (9*S*,10*R*) isomer of Z3,Z6,epo9–18 : H in both analyses utilizing reversed Chiralcel OJ-R and normal Chiralpak AD columns as shown in Figure 2[I] and [II], respectively. The chemical structure of the eluted epoxydiene was confirmed by its GC-MS data (Ando et al., 1993).

EAG Activity of Mulberry Looper Pheromone and Related Compounds. Figure 3[I] shows the EAG activity of Z3,Z6,epo9–18 : H and its related compounds in a racemic form. Male antennae of the mulberry looper responded most strongly to the pheromonal component among the tested 9,10-epoxydienes with a C_{17}–C_{23} chain and to 3,4- and 6,7-epoxydienes with a C_{18} chain. In this electrophysiological test with the optically pure enantiomers of Z3,Z6,epo9–18 : H, which were obtained by the chiral resolution in HPLC repeated three times, the activity of the (9*S*,10*R*) isomer was almost 100 times that of the (9*R*,10*S*) isomer as shown by their dose responses (Figure 3[II]).

Field Attraction of Mulberry Looper Males to Optically Active Pheromone. Results of the field tests in 1997 are shown in Table 2. Although damage by larvae of the mulberry looper was scarcely observed and the total number of captured male moths was not large in the mulberry field in Kanagawa Prefecture, Japan, most male moths were captured in traps baited with a 10 : 1 mixture of (9*S*,10*R*)-Z3,Z6,epo9–18 : H and racemic Z6,epo9–18 : H among the tested lures. When the (9*S*,10*R*)-epoxydiene was mixed with the enantiomer, the activity of this pheromonal component decreased. A mixture of (9*R*,10*S*)-Z3,Z6,epo9–18 : H and racemic Z6,epo9–18 : H did not attract males at all. In the mulberry field in Su Zhou, China, the population density of this species was very high and the pheromonal mixture including (9*S*,10*R*)-epoxydiene caught more than 60 males per trap in about three weeks. This field test also confirmed that the mixture including only the (9*R*,10*S*) isomer as the epoxydiene was inactive.

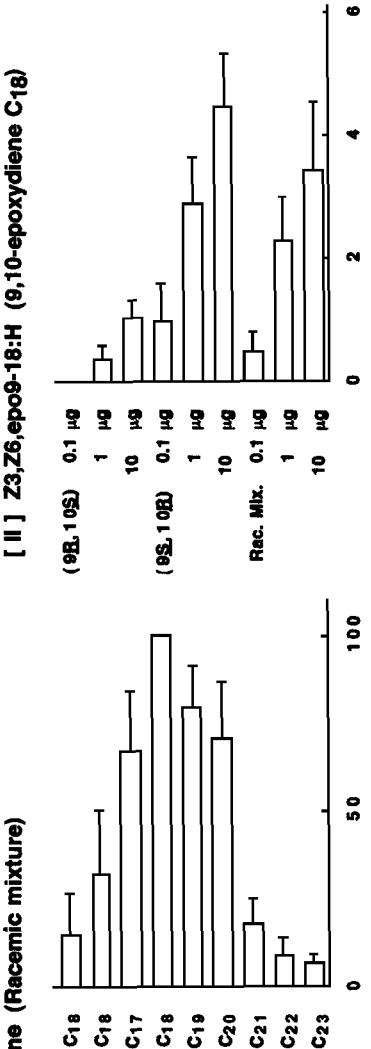

FIG. 3. EAG responses of mulberry looper males to the pheromonal epoxydiene and related compounds. [I] Relative activity (%) of each racemic epoxydiene (10 μg) based on the amplitude of racemic *Z3,Z6*,epo9–18:H (10 μg). [II] EAG amplitude (mV) of (9*R*,10*S*)-*Z3,Z6*,epo9–18:H, its (9*S*,10*R*) isomer and racemic mixture (0.1, 1 and 10 μg).

TABLE 2. FIELD TESTS WITH OPTICALLY ACTIVE EPOXYDIENE (Z3,Z6,epo9–18 : H) AND RACEMIC EPOXYMONOENE (Z6,epo9–18 : H) IN MULBERRY FIELDS IN KANAGAWA PREFECTURE, JAPAN, AND SU ZHOU, CHINA, IN 1997

Treatment (μg/septum)				Total captured males[a]	
Z3,Z6,epo9–18 : H		Z6,epo9–18 : H			
(9*R*,10*S*)-(+) isomer	(9*S*,10*R*)-(−) isomer	Racemate	Ratio	Japan[b] July 24–Oct 7	China[c] Sept 22–Oct 10
300	0	30	10:0:1	0 a	0 a
240	60	30	8:2:1	1 a	179 b
150	150	30	5:5:1	3 a	182 b
60	240	30	2:8:1	18 b	191 b
0	300	30	0:10:1	31 c	201 b
0	0	0		0 a	0 a

[a]Numbers within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple range test.
[b]Captured by two traps.
[c]Captured by three traps.

The isomer, however, did not inhibit the activity of the pheromonal epoxydiene, differing from the results in Japan.

DISCUSSION

HPLC with a chiral column is an ideal technique not only for analyzing the absolute configuration of a natural product but also for preparing optically active authentic samples of both enantiomers. Its application for research on lepidopterous sex pheromones is, however, still very limited, and there are few publications reporting the resolution of pheromonal *cis*-epoxydienes with normal-phase chiral columns (Chiralpak AD and AS) (Qin et al., 1997; Ando et al., 1997). We have obtained information on other chiral columns operated under different conditions and found a reversed-phase chiral column, Chiralcel OJ-R, packed with silica gel coated with a polysaccharide derivative [cellulose *tris*(4-methylbenzoate)] that separates enantiomers of epoxydienes, particularly 6,7-epoxydienes. Although enantiomeric separation of any 6,7-epoxydienes with a C_{17}–C_{23} chain was not sufficient by normal-phase chiral columns, this Chiralcel OJ-R column revealed excellent resolution as shown by chromatograms in Figure 1 and large values for separation factors (α = 1.17–1.26) in Table 1. Enantiomers of 3,4-epoxydienes with a C_{17}–C_{23} chain and 9,10-epoxydienes with a C_{17} or C_{18} chain are moderately separable by the OJ-R column (α = 1.07–1.10), as well as by the normal-phase columns.

The (+) enantiomers of 3,4- and 6,7-epoxydienes with a C_{17}–C_{23} chain

eluted faster than the (−) enantiomers from the Chiralcel OJ-R column. Optical rotations of epoxydienes stereoselectively synthesized by Sharpless epoxidation have been reported for C_{17}–C_{21} 3,4-epoxydienes (Cossé et al., 1992; Millar et al., 1990; Szöcs et al., 1993) and a C_{19} 6,7-epoxydiene (Mori and Ebata, 1986). All of the (3*S*,4*R*) and (6*S*,7*R*) isomers are dextrorotatory, and all of the (3*R*,4*S*) and (6*R*,7*S*) isomers are levorotatory. Therefore, it was expected that the shorter-R_t stereoisomers separated by the reversed-phase chiral column possessed the (*S*,*R*) configuration and the longer-R_t enantiomers possessed the opposite (*R*,*S*) configuration. In order to confirm their absolute configuration, we studied the stereochemistry of the hydrogenated products derived from optically active C_{20} epoxydienes. Because the hydrogenation over Pd-$BaSO_4$ proceeded without epimerization, and enantiomers of 3,4- and 6,7-epoxides with a saturated C_{20} chain could be also separated on the Chiralcel OJ-R column, each hydrogenated product was analyzed by this reversed-phase HPLC and compared with an authentic compound that was prepared via a Sharpless epoxidation reaction. The result verifies the elution pattern of these epoxydienes on the OJ-R column, and it is the same as that on normal-phase chiral columns, Chiralpak AD and AS (Qin et al., 1997). In contrast, (9*R*,10*S*) and (9*S*,10*R*) configurations of a C_{20} 9,10-epoxydiene were assigned for the shorter-R_t and longer-R_t enantiomers, respectively, by analysis of the hydrogenated products, contradicting the previous assignment determined by Mosher's method with MTPA esters, which were derived from methanolysis products of a C_{18} 9,10-epoxydiene (Qin et al., 1997). We examined again the stereochemistry of the C_{18} and C_{20} 9,10-epoxydienes by Mosher's method and realized the past mislabeling of enantiomers of the C_{18} compound. Therefore, we correct here the previous erroneous assignments and propose the exact elution order of C_{18}–C_{23} 9,10-epoxydienes on AS and AD columns starting from the dextrorotatory (9*R*,10*S*) isomers. This order is the same as that of C_{17} to C_{20} 9,10-epoxydienes on the OJ-R column.

Figure 4 shows the chemical structures of the stereoisomers that elute faster from the Chiralcel OJ-R column than the enantiomers. The (*S*,*R*) isomers of 3,4-, 6,7-, and 9,10-epoxyicosanes, which have shorter R_ts, have small alkyl groups on the left side and large alkyl groups on the right side in this figure, where the epoxy oxygen atom is set upward and behind the plane of this paper. We speculate that the enantiomer of a disubstituted epoxide with this configuration regularly elutes faster from the OJ-R column. The (*S*,*R*) isomers of 3,4- and 6,7-epoxydienes with a C_{17}–C_{23} chain, which also possess this configuration, invariably have shorter R_ts, indicating that their double bonds do not affect the elution order. The reason might be that the two double bonds of 3,4-epoxydienes are included in the right side large groups, and double bonds of the 6,7-epoxydienes are separately settled in the right and left side groups at the same β-position of the epoxy ring. In the case of 9,10-epoxydienes, double bonds have an influence on the elution order because the epoxy ring is located around the center

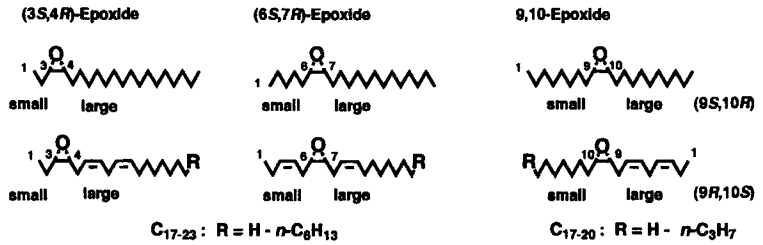

FIG. 4. Stereoisomers of *cis*-epoxides, which elute faster from a Chiralcel OJ-R column than the enantiomers.

of the molecule. Namely, the (*R*,*S*) isomers with a C_{17}–C_{20} chain eluted faster than the corresponding (*S*,*R*) isomers, and the enantiomers with a C_{21}–C_{23} chain could not be separated on the OJ-R column. Two homoconjugated double bonds prevent folding of the unsaturated C_8 chain, which might be larger than a saturated C_{10} chain and similar to a saturated C_{11}–C_{13} straight chain.

By a field test with both synthetic enantiomers (69% ee), Tan et al. (1996) estimated that the main pheromone component of the mulberry looper, *Z*3,*Z*6,epo9–18 : H, had an (9*S*,10*R*) configuration. Our trials with almost pure enantiomers that were prepared by preparative HPLC confirmed their results. The (9*S*,10*R*) isomer of *Z*3,*Z*6,epo9–18 : H showed stronger activity toward male moths in EAG and field tests than the enantiomer. Furthermore, examination of a pheromone extract from virgin female moths utilizing HPLC chiral columns proves the stereochemistry. A GC analysis is sensitive and generally suited for pheromone research, but enantiomeric separation with a capillary chiral GC column has not been reported for the 9,10-epoxydiene. Our studies indicate that HPLC with chiral columns is useful to directly determine the absolute configuration of an epoxy pheromone that includes a chromophore for UV detection such as a homoconjugated diene system. The mulberry looper secretes an epoxymonoene, *Z*6,epo9–18 : H, as a minor component, and its stereochemistry has also been estimated only by field tests (Tan et al., 1996). Analysis of the epoxymonoene in the pheromone extract has not been accomplished; thus the configuration produced by the female is unknown. While detection of a minor component by HPLC is very difficult, we are interested in its chromatographic behavior. In addition to this minor component, we are attempting to resolve many kinds of epoxymonoenes by HPLC on chiral columns. Results will be reported in the near future.

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OLFACTORY REACTIONS OF THE TWELVE-SPOTTED LADY BEETLE, *Coleomegilla maculata* AND THE GREEN LACEWING, *Chrysoperla carnea* TO SEMIOCHEMICALS RELEASED FROM THEIR PREY AND HOST PLANT: ELECTROANTENNOGRAM AND BEHAVIORAL RESPONSES

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Abstract—Electroantennograms (EAGs) were recorded from two predatory insect species, the twelve-spotted lady beetle, *Coleomegilla maculata* and the green lacewing, *Chrysoperla carnea* in response to semiochemicals emitted from one of their prey species, the pea aphid *Acyrthosiphon pisum* and their host plant. EAG responses were also recorded from *C. maculata* in response to extracts from individuals of the opposite sex and to extracts from an herbaceous plant, catnip *Nepeta cataria*. Extracts of catnip and two sex pheromone components of aphids, (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol, elicited significant EAG responses from the antennae of both predatory species. Of 10 corn volatile compounds tested, *C. carnea* adults responded most strongly to 2-phenylethanol and (*E*)-β-farnesene. A significant difference in EAG response to extracts of corn leaf collections was observed between male and female *C. carnea*. In *C. maculata*, significant EAG responses were elicited by most of the tested corn volatile compounds, except α-pinene and (*E*)-2-hexenal. The highest EAG responses were observed in response to (*E*)-β-farnesene,

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α-terpineol, 2-phenylethanol, and β-caryophyllene. Sexual differences in EAG responses of *C. maculata* were only found in response to 1-octen-3-ol. Male antennae of *C. maculata* produced significant EAG responses to extracts from conspecific females, but not to males, which indicates that some chemicals from females could be involved in sexual communication. A significant EAG response also was recorded in response to the extracts of fluids produced during "reflex bleeding." Male and female antennae of both species exhibited similar dose–response curves to most of the selected compounds, although female *C. maculata* antennae exhibited higher thresholds in response to several compounds including α-terpineol, (*Z*)-3-hexenol, and (4a*S*,7*S*,7a*R*)-nepetalactone. Field tests showed that 2-phenylethanol was highly attractive to both sexes of the two investigated species. Only *C. maculata* was attracted to traps baited with α-terpineol.

Key Words—Electroantennograms, dose–response, trapping test, corn volatile compound, aphid sex pheromone, catnip, aphid alarm pheromone, *Coleomegilla maculata*, Coccinellidae, *Chrysoperla carnea*, Chrysopidae.

INTRODUCTION

The use of predatory insects, including coccinellids and chrysopids, as biological control agents to suppress pest populations on either economically important crops or in home gardens is widely accepted and recognized by the general public and biological control practitioners (see references in Obrycki and Kring, 1998; Canard et al., 1984). There have been significant successes in using coccinellids and chrysopids to suppress whitefly, aphid, mealybug, scale, and mite populations (Gerling, 1990; Frazier, 1988; New, 1975). Despite the significant use of these two groups of predatory insects for biological control, very little is known about the mechanisms involved in prey location (van Emden and Hagen, 1976; Hagen, 1987). In particular, it is not known whether the predators are able to use odors directly from their prey or their host plant to eventually locate their prey. Because of the dispersal behavior of many aphidophagous species and their polyphagous nature (Frazier, 1988), attractive compounds could be useful in retaining predaceous insects in fields where they were released.

The twelve-spotted lady beetle, *Coleomegilla maculata* (Degeer) (Coleoptera: Coccinellidae), and the golden eyed green lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), are two of the most common aphid predators found in field crops in the eastern United States. They are especially abundant in crops such as cotton, corn, and alfalfa. In corn these two predatory species feed on aphids and European corn borers (Obrycki and Kring, 1998; Phoofolo, 1997; Sparks et al., 1966; Udayagiri, 1996), and in alfalfa they feed on aphids and alfalfa weevil larvae (Giles et al., 1994). In the present study we report results of electroantennogram (EAG) and behavioral responses of *C. carnea* and *C. maculata* to selected semiochemicals released from corn leaves and from an

aphid prey, the pea aphid *Acyrthosiphon pisum.* (Homoptera: Aphididae). The possible existence of sex pheromone compounds in *C. maculata* was also investigated.

METHODS AND MATERIALS

Insects. Coleomegilla maculata adults were collected from an overwintering site in Roland, Iowa. Larvae of *C. carnea* were purchased from Rincon-Vitova, California, and reared at 20°C and 16L : 8D period. Females were mated and produced eggs. Larvae were fed with pea aphids *A. pisum* and frozen *Ephestia kuehniella* eggs until pupation. Adults were sexed and kept in separate cages until used in experiments.

Extraction of Potential Sex Pheromone-like Substances from C. maculata. Adults of both sexes were anesthetized by placing them at −20°C, and the abdomen and elytra of adults were cut off with a pair of fine scissors. Dissecting tools were rinsed with acetone several times between dissections to avoid cross-contamination. The dissected parts were extracted in a 1-ml vial containing 500 μl of hexane for 2 hr. Ten males and 10 females were dissected and extracted. The extracts were concentrated to 200 μl under a mild stream of N_2. Approximately 20 μl of the extracts, equivalent to one adult, were used for the EAG tests.

Collection of Defensive Compound Extracts. Both sexes of *C. maculata* were handled vigorously and pressed against a piece of filter paper (Whatman, No. 1), resulting in reflex bleeding production of orange-colored fluid excreted through the joints of the legs. The fluid was absorbed into filter paper; fluid from 20 beetles was collected. The filter paper used for the collection was then cut into small pieces and placed in a 2-ml vial and extracted with 200 μl of hexane for 1 hr. A volume of 10 μl of the concentrated extract was used for EAG tests.

Collection of Plant Volatiles. Corn (Pioneer, Hybrid 3489) was grown in the greenhouse under approx. 25°C and 75% humidity, with natural light supplemented by 400-W high-pressure sodium lights. The three-leaf stage of plants was used for volatile collections because this life stage is infested by corn leaf aphids (Bing et al., 1992), and it is also a stage searched by these predatory species. Corn leaves were removed from plants, and ca. 15 g placed in a 0.5-liter glass collector with two openings on each side. A stream of purified air passing through a freshly activated charcoal filter was blown into the inlet of the collector, through the corn leaves, and exited through the outlet connected to a Tenax trap. The trap consisted of a Pasteur pipet (5 cm long and 0.5 cm diameter) packed with 300 mg of Tenax (20–35 mesh, Alltech, Illinois). The flow rate of the air was 150 ml/min from the outlet of the Tenax trap, and the volatiles were collected for 18 hr. The trapped volatiles were eluted with HPLC-grade hexane.

The extracts from three collections were combined and concentrated to 100 μl. Ten microliters of extracts were used for each EAG test.

Extracts of Catnip. Dried seeds and flowers of catnip *Nepeta cataria* were purchased from a local pet store. For EAG tests, approx. 0.2 mg of dried plant material was homogenized and extracted by adding 500 μl of hexane. The extract was poured through a funnel with filter paper (Whatman No. 1), and the residue was discarded. The extract was concentrated to 100 μl under a mild stream of N_2. The extract was stored at −20°C until used in EAG tests.

Electroantennogram Responses. EAG recordings were made by connecting an electrogel-filled (Spectro 360, Parker Laboratory, New Jersey) glass pipet Ag–AgCl electrode to the excised head from a beetle or a lacewing. A recording electrode filled with the same electrogel connected to a high-impedance DC amplifier with automatic baseline drift compensation was placed in contact with the tip of the antennae. Antennal responses to various chemical stimuli were first recorded for a series of commercial synthetic compounds representing known corn leaf volatiles. Then, male and female body extracts of *C. maculata*, reflex bleeding extracts, aphid alarm pheromone, and catnip extracts were tested. A second series of EAG tests was then conducted including a dose–response series with selected EAG-active compounds from the first test. Serial dilutions of the tested compounds were made in redistilled HPLC-grade hexane at dosages ranging from 0.1 to 1000 μg, except 2-phenylethanol, for which methylene chloride was used as the solvent. The tested compounds and extracts were applied to filter-paper strips (0.5 × 2.5 cm, Whatman No. 1) in 10 μl of solvent, except as otherwise noted. The filter-paper strips were inserted into Pasteur pipets (15 cm long). Control puffs of air were applied after each puff of a test stimulus. The response to the air puff was used to normalize beetle or lacewing responses relative to the tested stimuli. The average EAG response to the air puff from antennae of *C. maculata* and *C. carnea* was 0.05 ± 0.003 mV ($N = 30$) and 0.02 ± 0.001 mV ($N = 27$), respectively. The relative EAG response of each stimulus was normalized by dividing the amplitude of the test stimulus by the mean of the response to the control puff. The sequence of exposure of each stimulus to each antenna was randomly defined.

Chemicals. Ten corn volatile compounds were used, and the source and purity of these compounds are listed in Table 1. The two aphid sex pheromone components, (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol were kindly provided by Dr. John Pickett of the IACR-Rothamsted, UK. The purity of these two compounds was approx. 97% and 55%, respectively, as analyzed by GC-MS.

Field Test. A preliminary field trapping test was conducted in an alfalfa field in Ames, Iowa. Synthetic compounds were prepared in hexane or methylene chloride. Medical peerless cotton rolls (5 cm long, 100% cotton) impregnated with a dose of 50 mg of each compound were used as dispensers, and the traps

TABLE 1. SOURCE AND PURITY OF CORN VOLATILE COMPOUNDS USED IN EAG TESTS

Compound	Source of supply	Purity (%)[a]
(*E*)-β-Farnesene	Bedoukian Research Inc.	>99
α-Terpineol	Bedoukian Research Inc.	>99
α-Pinene	Aldrich Chemical Co.	95
(*Z*)-3-Hexenal	Bedoukian Research Inc.	>99
2-Phenylethanol	Bedoukian Research Inc.	>99
(*E*)-2-Hexenal	Sigma Chemical Co.	>99
β-Caryophyllene	K & K Laboratories	>98
1-Hexanol	Sigma Chemical Co.	98
(*Z*)-3-Hexenol	Bedoukian Reserach Inc.	>99
1-Octen-3-ol	Bedoukian Research Inc.	>99

[a]Label information

used were similar to the Rebell Trap (Great Lakes IPM, Michigan). The trap was hung from the bent end of an electric farm post, 1.2 m above the ground. Within a replicate (N = 3), traps were set at least 10 m apart. The traps were checked daily, and trap position within a series was randomized to minimize the effects of habitat heterogeneity.

Statistics. The resulting EAG data (means of relative responses) and trap catches (means of trapped species) were compared by analysis of variance followed by Fisher's protected least significant difference test (FPLSD).

RESULTS

Selectivity. In general, the antennae of both sexes of the two predatory species, *C. maculata* and *C. carnea*, responded strongly to several green leaf volatiles and sex pheromones of their aphid prey. No obvious differences in the sizes and morphologies of their receptor organs were observed between the two sexes of the same species. Within each species, slight differences in EAGs to each of the tested stimuli were noted, but only two of them were statistically different.

Response to Crude Extracts. In *C. maculata*, female extracts of both the whole abdomen and the elytra elicited higher EAG responses from male antennae compared with those of male extracts (Figure 1). The extracts from the defensive reflex bleeding also elicited EAG responses. Both male and female antennae responded strongly to catnip extracts (Figure 2). In *C. carnea*, no significant EAG responses were observed when stimulated with the extract of *C. maculata* reflex bleeding (data not shown here), but significant EAG responses were elicited when tested with catnip extracts (Figure 3). EAG response of female *C. carnea* antenna to extracts of corn leaves was greater than that of the males.

Response to Corn Leaf Volatiles. Ten common corn leaf volatiles, previously

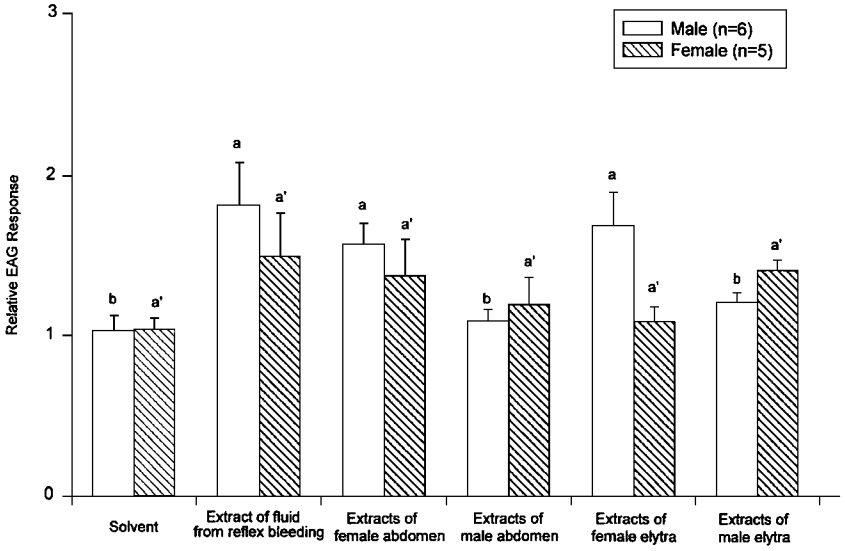

FIG. 1. EAG responses of male and female *Coleomegilla maculata* to various extracts from conspecific body parts and the fluid of reflex bleeding. Means (± standard error) with no letters in common are significantly different (ANOVA followed by FPLSD test, $P < 0.01$).

identified from corn leaves (Buttery and Ling, 1984), were selected for a standard EAG test. In both sexes of *C. maculata*, antennae responded to most of the tested compounds. Responses to (*E*)-β-farnesene, α-terpineol, and 2-phenylethanol were significantly higher than those to α-pinene of (*E*)-2-hexenal (Figure 2). However, a significantly higher EAG response was elicited by 1-octen-3-ol from male antenna, relative to that from a female (Figure 2). In *C. carnea*, only two corn volatile compounds elicited significant EAG responses, with a significant response from 2-phenylethanol and (*E*)-β-farnesene, respectively (Figure 3).

Response to Sex Pheromone and Alarm Pheromone of Aphids. Both sexes of the two investigated species responded to sex pheromone components used by their prey species, (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol. In *C. maculata*, a relatively higher EAG response was elicited by (4a*S*,7*S*,7a*R*)-nepetalactone than that by the alcohol (Figure 2). Aphid alarm pheromone, (*E*)-β-farnesene, elicited EAG responses from male antennae as high as those in response to aphid sex pheromone. In *C. carnea*, there were no significant differences between male and female EAGs to aphid sex pheromone components (Figure 3). However, female antennae of *C. carnea* responded to (4a*S*,7*S*,7a*R*)-nepetalactone greater than to (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol.

Sensitivity. EAG responses in *C. maculata* generally increased with increas-

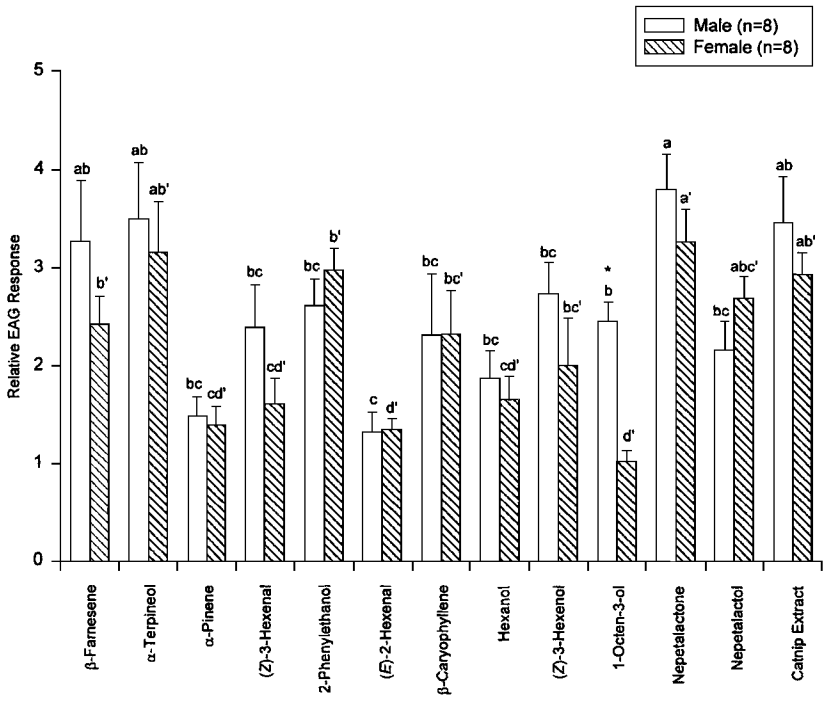

FIG. 2. EAG responses of male and female antennae of *Coleomegilla maculata* to several synthetic corn leaf volatile compounds, sex pheromone components of aphids and the crude extract of catnip. Means (± standard error) with no letters in common are significantly different (ANOVA followed by FPLSD test, $P < 0.05$). An asterisk above the letter indicates a significant difference in EAGs between male and female antennae in response to this compound (Student's t test, $P < 0.001$).

ing doses of the selected synthetic corn volatile compounds (Figure 4). EAG dose–response curves showed that male antennae reached saturation at the dose of 100 μg for two of the tested corn volatile compounds, (*E*)-β-farnesene and α-terpineol (Figure 4). Female antennae appeared to be relatively sensitive to α-terpineol and 2-phenylethanol, with significant EAG responses elicited at a dose of 10 μg. In response to (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol, the minimum dose for a significant EAG response from both male and female antennae was 100 μg, and saturation likewise occurred at this same dose (Figure 4).

In *C. carnea*, response curves of both male and female antennae in response to the tested corn volatile compounds exhibited significant differences in response sensitivity (Figure 5). EAG responses of both sexes to (*E*)-β-farnesene increased

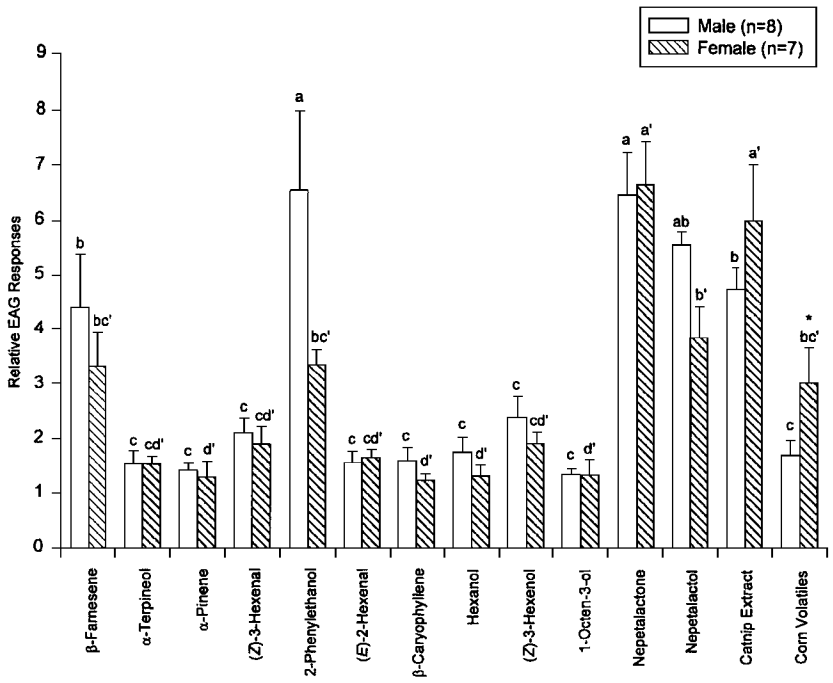

FIG. 3. EAG responses of male and female antennae of *Chrysoperla carnea* to several synthetic corn leaf volatile compounds, sex pheromone components of aphids and the crude extract of catnip. Means (± standard error) with no letters in common are significantly different (ANOVA followed by FPLSD test, $P < 0.05$). An asterisk above the letter indicates a significant difference in EAGs between male and female antennae in response to this compound (Student's t test, $P < 0.05$).

with increasing doses, and the highest EAG response was observed at a dose of 1000 μg. Significant EAG responses were elicited from the antennae of both sexes to 2-phenylethanol at 100 μg dosage. No significant differences in EAG responses among the three higher tested doses of this compound were observed. In contrast, α-terpineol and (*Z*)-3-hexenol from corn elicited relatively lower EAG responses at all dosages tested. For the two aphid sex pheromone components, the same trends were observed as those shown in *C. maculata*. Saturation appeared at a dose of 100 μg for both compounds in both sexes.

Field Tests. The results from our preliminary field trapping test showed that both species were attracted to one of tested corn volatile compounds, 2-phenylethanol (Figure 6). In addition, still higher catches of *C. maculata* were observed in traps baited with α-terpineol.

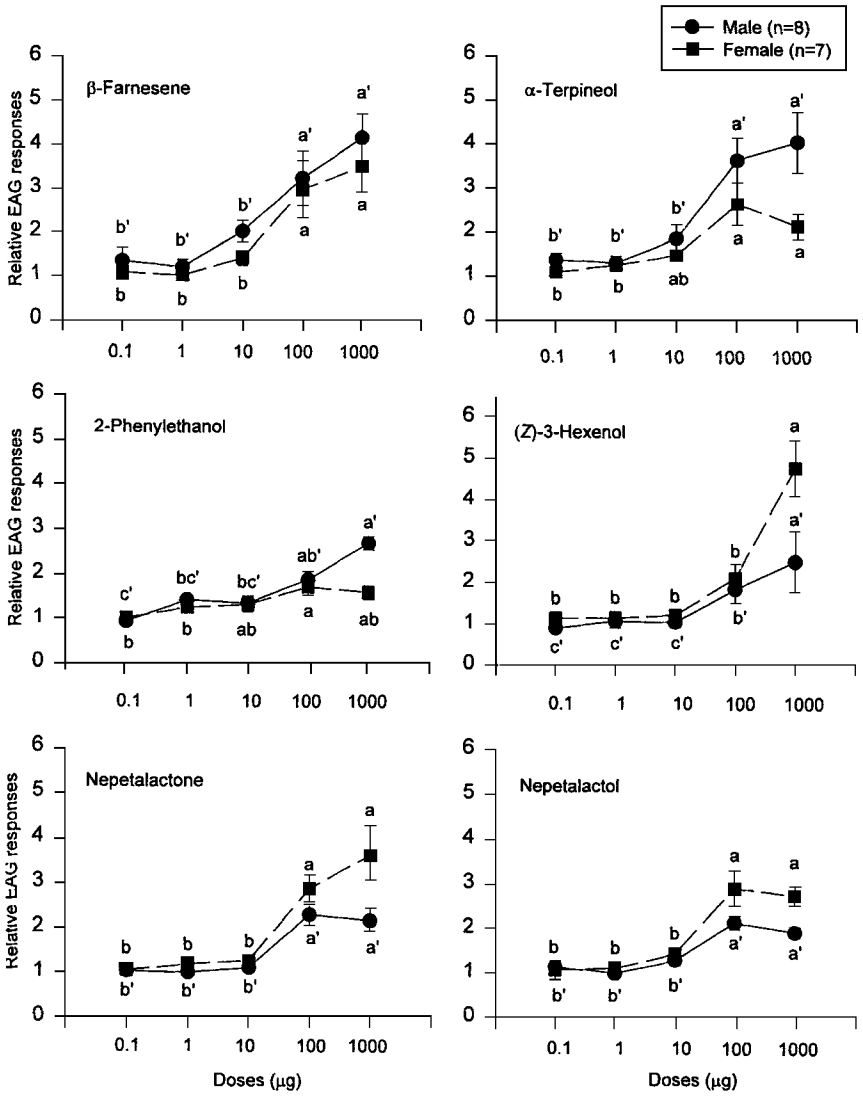

FIG. 4. Dose–response curves constructed from EAGs of male and female of *Coleomegilla maculata* to four synthetic corn leaf volatile compounds and two sex pheromone components of aphids. Means (± standard error) of different dosages among the same stimulus with no letters in common are significantly different (ANOVA followed by FPLSD test, $P < 0.05$).

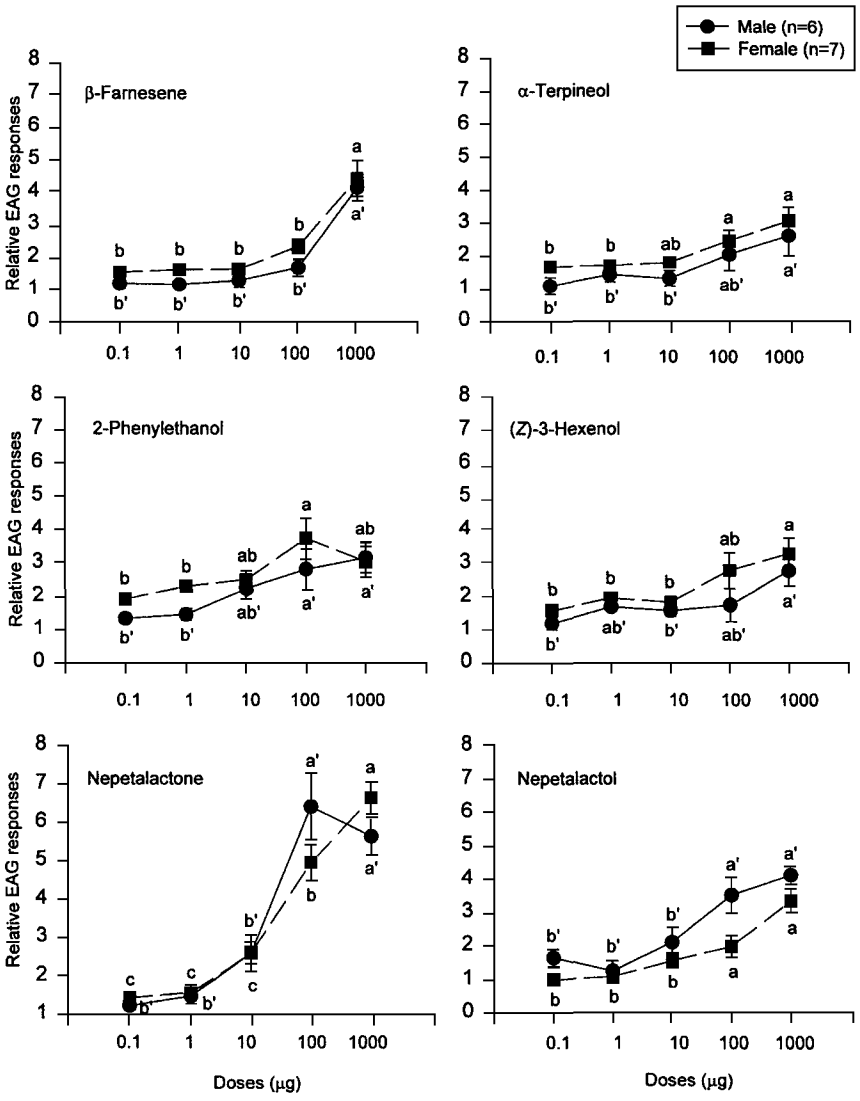

FIG. 5. Dose–response curves constructed from EAGs of male and female of *Chrysoperla carnea* to four synthetic corn leaf volatile compounds and two sex pheromone components of aphids. Means (± standard error) of different dosages among the same stimulus with no letters in common are significantly different (ANOVA followed by FPLSD test, $P < 0.05$).

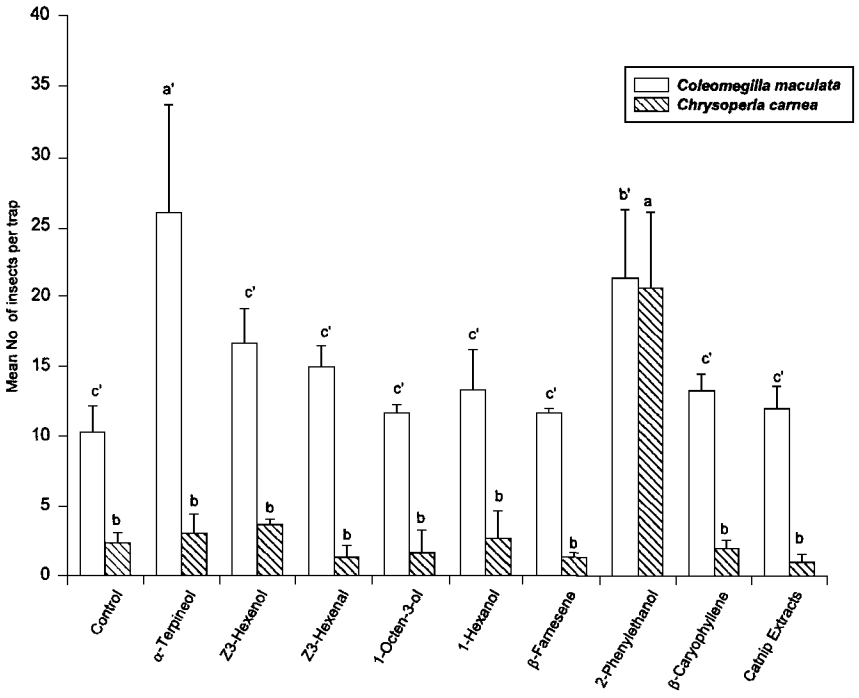

FIG. 6. Mean number of *Coleomegilla maculata* and *Chrysoperla carnea* caught in traps baited with corn volatile compounds (50 mg) and crude extracts of catnip in the alfalfa field (line indicates SE) (± standard error). For each species, columns with no letters in common are significantly different (ANOVA followed by FPLSD test, $P < 0.05$).

DISCUSSION

To our knowledge, this is the first published report of successful EAG recordings from the two predatory insect species, *C. maculata* and *C. carnea*. The significant EAG responses to semiochemicals released from their potential prey species and their host plants indicate that these two predators could possibly use such chemicals to locate their prey. These EAG-active compounds, if they prove to have behavioral activities as attractants or arrestants, could possibly be used to enhance the efficacy of these two predaceous insects in pest management systems. Emitting these compounds in cropping systems could attract lady beetles and lacewings to their target habitats and retain them in habitats for longer durations.

Volatiles from corn plants contain general and specific odor components including green leaf alcohols, aldehydes, derivative esters, terpenes, and

sesquiterpenes (Buttery and Ling, 1984). Many parasitoids use semiochemicals associated with the host or the host habitat to locate their prey (Udayagiri, 1996; Turlings et al., 1992). In coccinellids, only Obata (1987) has reported that chemical cues could be involved in prey finding. EAG responses from the antennae of *C. maculata* elicited by corn volatile components including (*E*)-β-farnesene, α-terpineol, 2-phenylethanol, β-caryophyllene, (*Z*)-3-hexenol, hexanol, and 1-octen-3-ol demonstrate that this predator could possibly respond to odors from host habitats for prey location. Our preliminary field trapping results showed that adult *C. maculata* were attracted to at least two components, α-terpineol and 2-phenylethanol. Approximately equal numbers of both sexes were caught in traps.

Among the seven corn volatile compounds found to be EAG-active in *C. maculata*, (*E*)-β-farnesene has also been reported as an aphid alarm pheromone component (Nault et al., 1973). This compound is emitted from aphids when they are disturbed. The perception of this pheromone by aphids leads to dispersal from their feeding sites. Results of our preliminary behavioral tests show that two lady beetle species, *Hippodamia convergens* and *Harmonia axyridis*, are attracted by this compound (data not shown here). This compound, however, does not elicit searching behaviors in another lady beetle, *Coccinella septempunctata* (Nakamuta, 1991). The active EAG response recorded from the antennae of *C. maculata* to this compound could also be a result of responding to corn volatiles, because (*E*)-β-farnesene is also a common compound among corn leaf volatiles, although it is generally found together with other sesquiterpene hydrocarbons. Colburn and Asquith (1970) have showed that odors from mites feeding on plants, including (*E*)-β-farnesene, are used by adults of the ladybird beetle *Stethorus punctum* to select host plants. However, no differences in catches were found from the traps baited with this compound compared to those of the control.

Chemicals secreted by aphids other than (*E*)-β-farnesene, such as breakdown products of honeydew, are known to cue local search by lady beetles (Evans and Richards, 1997). Sex pheromone communication in mate finding was demonstrated in aphids in the genus *Schizaphis* (Pettersson, 1970). Virgin females (oviparae) release a male-attracting pheromone from scent plaques on their metathoracic tibiae. Dawson et al. (1989) identified sex pheromones of several aphid species as a combination of (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol. It has been suggested that female parasitoids may use this aphid sex pheromone as a kairomone to locate females of their holocyclic aphid hosts (Hardie et al., 1994). The present study shows that antennae of adult *C. maculata* responded with significant EAGs to these two components of aphid sex pheromone, which indicates that *C. maculata* may use them or similar compounds in prey finding. Significant EAG responses were also observed from the antennae of *C. maculata* in response to the extract of catnip, which may be explained because (4a*S*,7*S*,7a*R*)-nepetalactone is a major constituent in essential oil of *N. cataria* (Eisenbraun et al., 1980).

Because adult *C. carnea* are reported most active at twilight or during the night (Duelli, 1984) when the use of visual cues might be impaired, significant EAG responses to plant volatile compounds and sex pheromones of their prey species suggest that chemical orientation may be involved in prey host location. Van Emden and Hagen (1976) found that tethered *C. carnea* females responded positively in flight toward isomers of tryptophan and their breakdown products. Our results show that the antennae of *C. carnea* are highly sensitive to two corn leaf volatiles, (*E*)-β-farnesene and 2-phenylethanol. However, in the field trapping test *C. carnea* adults were only attracted to the latter compound. Both sexes of lacewings were caught in the traps baited with 2-phenylethanol. Female catches were significantly higher than males (data not shown here). The recorded EAG response to (*E*)-β-farnesene from *C. carnea* is different from the results obtained from the Asian lacewing *Chrysopa cognata* (Boo et al., 1998). Significant EAG responses elicited by 2-phenylethanol at a relatively lower dose may indicate the ability of *C. carnea* to detect the compound at a greater distance from its source. Further behavioral studies to test this hypothesis are being conducted.

An earlier study showed that β-caryophyllene acts as an attractant to adults of *C. carnea* in the field (Flint et al., 1979). However, our present EAG study showed a relatively low EAG response obtained from the antennae of *C. carnea* and low catches of *C. carnea* in a field test. The significant EAG responses from *C. carnea* to sex pheromones of their prey aphid species presented here suggest that both males and females could possibly locate their prey by responding to these compounds. Recently Boo et al. (1998) reported that adult *C. cognata* are attracted to (4a*S*,7*S*,7a*R*)-nepetalactone and mixtures of this compound with (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol in the field.

The search for sex pheromones in Coccinellidae during the last decade has not been successful. However, recent studies on the ultrastructure of integumentary glands in the coccinellid *Semiadalia undecimnotata* as well as specific olfactory sensilla types clustered on the two distal segments of the antennae may indicate the possible involvement of long-range chemosensory perception (Barbier et al., 1992; Jourdan et al., 1995). In our present EAG experiments, we demonstrated that the female extracts of *C. maculata* elicited significant EAG responses from male conspecific antennae, which suggest that the substances from females could be used as sex pheromone for mate finding.

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FURTHER ALKALOIDS COMMON TO ANTS AND FROGS: DECAHYDROQUINOLINES AND A QUINOLIZIDINE

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Abstract—Three alkaloids—two minor decahydroquinolines (DHQs) and a major quinolizidine—were detected in an extract of a Brazilian myrmicine ant (*Solenopsis* (*Diplorhoptrum*) sp. *picea* group). One DHQ (**3**) was identical to a known frog-skin alkaloid, *cis*-**195A** (*cis*-5-methyl-2-propyldecahydroquinoline), while the second DHQ, an isomer of **3**, designated **195J**, was assigned a tentative *cis*-2-methyl-5-propyldecahydroquinoline structure (**2**) based on mass and infrared spectra. The third alkaloid proved identical to the frog-skin alkaloid **195C**, for which a structure had not been previously proposed. Mass and infrared spectral analysis, including chemical ionization tandem mass spectrometry, indicated a 4-methyl-6-propylquinolizidine structure (**1**) for **195C**. The four possible diastereomers were synthesized and the (6*Z*,10*E*)-4-methyl-6-propylquinolizidine diastereomer (**1b**) was identical to the natural alkaloid. Skin extracts of a population of a Madagascan mantelline frog contained, among other alkaloids, minor amounts of the same alkaloid

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triad **1–3** with **1** again predominating. The common occurrence of alkaloids **1–3** in both ant and frog supports the hypothesis that ants are a likely dietary source for sequestered frog-skin alkaloids and brings to six, the alkaloid classes common to ant and frog.

Key Words—Alkaloids, mass spectrometry, infrared spectroscopy, amphibians, ants, decahydroquinolines, quinolizidines.

INTRODUCTION

A variety of saturated nitrogen heterocycles have been found as venom components in species of the myrmicine genus *Solenopsis (Diplorhoptrum)*, commonly known as thief ants. These include 2,5-dialkylpyrrolidines, 2,6-dialkylpiperidines, 3,5-dialkylpyrrolizidines, and 3,5-dialkylindolizidines (Jones et al., 1996 and references therein). A pair of 2,5-disubstituted decahydroquinolines were recently reported in extracts of virgin queens of a *Solenopsis (Diplorhoptrum)* ant from Puerto Rico (Spande et al., 1998). In this report, we describe the first isolation from an animal source of a 4,6-dialkylquinolizidine, namely (6*Z*,10*E*)-4-methyl-6-propylquinolizidine (Figure 1, **1b**), as a major alkaloid in workers of a *Solenopsis (Diplorhoptrum)* species obtained near Itabuna, Brazil. In addition, smaller amounts of a *cis*-fused 2-methyl-5-propyldecahydroquinoline (**2**) and the isomeric 5-methyl-2-propyldecahydroquinoline (**3**) were detected.

The rich array of alkaloids, detected over the past three decades in skin extracts from certain dendrobatid, mantelline, myobatrachid, and bufonid anurans (Daly et al., 1993), is now thought to originate from dietary sources (Daly et al., 1992, 1994a,b, 1997), which for such frogs/toads consist of small, often tiny, arthropods. Some of the alkaloid-containing dendrobatid frogs have been proposed to be "ant specialists" (Toft, 1980, 1995; Donnelly, 1991; Caldwell, 1996), and, including the present work, representatives of six of the approximately 20 major structural classes of alkaloids detected in extracts of frog skins have now been identified in myrmicine ants, especially *Solenopsis (Diplorhoptrum)* species (Jones and Blum, 1983; Daly, 1995; Spande et al., 1998).

The identification of quinolizidine **1b** has made it possible to assign this structure to alkaloid **195C**, previously detected in many frog skin extracts but whose structure had not been established. In addition, the decahydroquinolines **2** and **3** were shown to correspond to alkaloids present in frog skin extracts. These results reinforce the dietary hypothesis for amphibian alkaloids since the same mixture of **1b**, **2**, and **3** has been found in the Brazilian *Solenopsis (Diplorhoptrum)* species and in frogs of the species *Mantella betsileo*.

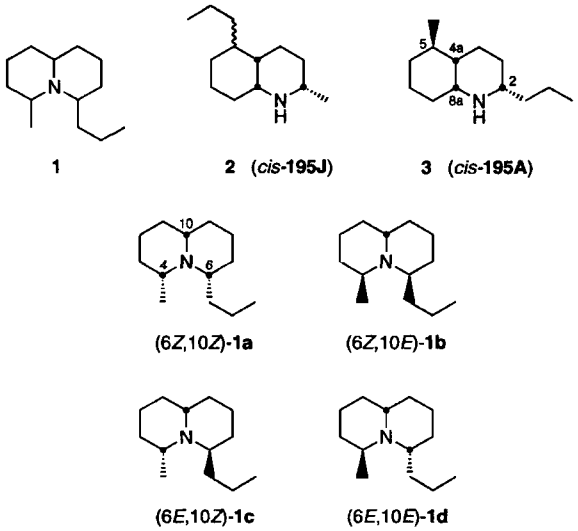


FIG. 1. Structures of **1a–1d**, **2**, and **3**.

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic-mass spectral analyses were performed with Hewlett-Packard model 5890 gas chromatograph equipped with a 30-m × 0.32-mm Rtx-5 column. Vapor-phase FTIR spectra were obtained from a Hewlett-Packard model 5965B detector interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a 30-m × 0.25-mm Rtx-5 Amine column. IR spectra of liquids were obtained with a Perkin-Elmer 1600 series FTIR instrument. Mass spectra were obtained in the EI mode from a Shimadzu QP-5000 GC-MS equipped with a 30-m × 0.32-mm Rtx-5 column. High-resolution mass spectrometry (HR-MS) was performed with a JEOL SX102 instrument equipped with a 15-m × 0.20-mm HP-5 column. EI-MS, EI-MS/MS and CI-MS/MS (NH_3) data were obtained with a Finnigan GCQ instrument equipped with a 25-m × 0.25-mm Rtx-5 column.

Ants. Two collections of *Solenopsis (Diplorhoptrum)* sp. *picea* group were made near Itabuna, Bahia, Brazil, and immediately placed in small vials containing methylene chloride. Voucher specimens were deposited in the collection of the Los Angeles County Museum of Natural History, Los Angeles, California.

Frogs. Collections of mantelline frogs, including three populations of *Mantella betsileo* Grandidier 1872, were made in Madagascar (Garruffo et al., 1993a; Daly et al., 1996). The *Mantella betsileo* populations were from near Antanambaobe (11 skins, December 1990), Ambavala (8 skins, January 1994), and Farakaraina (12 skins, December 1993). Skins were extracted with methanol,

and alkaloid fractions were obtained as described (Garraffo et al., 1993a; Daly et al., 1994a). Quinolizidine **195C**, a major alkaloid in skin extracts of *Mantella betsileo* from Antanambaobe and Ambavala, was isolated from the combined alkaloid fractions from these two populations on a reverse-phase column with a linear gradient of CH_3CN–H_2O (10 : 90 to 90 : 10; cf. Garraffo et al., 1997). Voucher specimens are in the collection of the American Museum of Natural History (New York City).

Synthesis (see Scheme 1)

2-[3-(6-Propyl-2-pyridyl)-propyl]-1,3-dioxolane (*5*). A solution of the anion derived from 1.0 g (5 mmol) of 2-[3-(6-methyl-2-pyridyl)-propyl]1,3-dioxolane (**4**) (Jones et al., 1996) in 25 ml of diethyl ether and 4.0 ml of 1.3 M 2-butyllithium in hexanes, was added to 0.7 ml (7 mmol) of iodoethane in 15 ml of diethyl ether. The mixture was stirred 1 hr, then worked up in the usual manner to provide 1.1 g of **5** that was >90% pure by gas chromatographic analysis. GC-FTIR: 3066, 2959, 2882, 1580, 1454, 1401, 1249, 1140, 1052, 940 cm^{-1}. MS, *m/z* (rel %): 235(M^+, 2), 234(2), 207(10), 190(13), 164(10), 162(28), 136(10), 135(100), 107(10), 73(29), HR-MS *m/z*: calcd for $C_{14}H_{21}NO_2$, 235.1572; observed, 235.1589.

cis-*2-[3-(6-Propyl-2-piperidyl)-propyl]-1,3-dioxolane* (*8*). A solution of 1.018 g of dioxolane **5** and 0.5 g of Rh-on-alumina catalyst (5%) in 50 ml of ethanol was hydrogenated at 3 atm for 4 hr. After filtration, the solvent was removed in vacuo. GC-MS analysis of the residue showed it to be homogeneous. GC-FTIR: 2934, 2881, 2800, 1453, 1400, 1331, 1137, 1055, 941 cm^{-1}. MS, *m/z* (rel %): 240(M^+-1, 2), 198(18), 138(15), 136(14), 127(10), 126(100), 96(9), 84(10), 73(24). HR-MS *m/z*: calcd for M^+, $C_{14}H_{27}NO_2$, 241.2042; observed, 241.2036.

cis- *and* trans *2-[3-(6-Methyl-2-piperidyl)-propyl]-1,3-dioxolane* (*6 and 7*). For the sodium-in-ethanol reduction, a solution containing 2.0 g (10 mmol) of pyridine acetal **4** in 80 ml of absolute ethanol was stirred and heated to reflux under a drying tube and 16 g of sodium metal were added in small pieces over 45 min. The mixture was stirred at reflux for 3 hr, then hydrolyzed by slowly adding several volumes of water. The solvent was removed in vacuo, and the organic residue was extracted with 3 × 25 ml of diethyl ether. The total ether extract was dried over anhydrous K_2CO_3, filtered, and the solvent removed in vacuo. The resulting crude liquid was Kugelrohr-distilled at reduced pressure to give 1.5 g of a colorless oil. GC-MS analysis showed the presence of two components (**6** and **7**) with identical mass spectra in a 1 : 4 ratio. The isomers were separated by column chromatography by using 200 g of alumina and a solvent gradient as previously described (MacConnell et al., 1971) to give 0.10 g of the first eluting isomer (**6**) and 0.34 g of the second eluting isomer (**7**). GC-FTIR of

cis-piperidine **6**: 2934, 2881, 2802, 2718, 2605, 1438, 1400, 1325, 1137, 1056, 940, 819 cm^{-1}. GC-FTIR of *trans*-piperidine **7**: 2935, 2882, 2654, 1456, 1400, 1378, 1263, 1138, 1057, 940, 813 cm^{-1}. Typical MS, *m/z* (rel %): 212(M^+-1, 1), 99(8), 98(100), 82(4), 81(4), 73(19). HR-MS *m/z*: calcd for M^+-1, $C_{12}H_{22}NO_2$, 212.1651; observed, 212.1663; calcd for $C_6H_{12}N$, 98.0970; observed, 98.0973.

For catalytic hydrogenation, a solution of 1.90 g (9 mmol) of dioxolane **4**, 15 drops of triethylamine, and 0.7 g of Rh-on-alumina catalyst in 50 ml of ethanol was hydrogenated at 3 atm for 3 hr. After filtration, the solvent was removed in vacuo. Diethyl ether was added to the residue, and the solution was washed with water and dilute NaOH. The ether layer was dried over anhydrous K_2CO_3, filtered, and the solvent was removed in vacuo to give 1.85 g of a colorless oil. GC-MS analysis showed that this oil was homogeneous and had a mass spectrum and retention time identical to that of *cis*-piperidine **6**.

4-Methyl-6-propyl-quinolizidines (***1a–1d***). For derivation from a mixture of piperidines **6** and **7**, a solution containing the mixture of piperidines **6** and **7** from a sodium-in-ethanol reduction of 5 g (25 mmol) of **4** in 70 ml of freshly distilled CH_2Cl_2 was treated with an aqueous solution of KCN. The pH was adjusted to 6 with dilute HCl, and the mixture stirred overnight. The organic layer was separated, dried over anhydrous K_2CO_3, filtered, and the solvent was removed in vacuo to give 3.6 g of a liquid. A solution of 0.19 g (1 mmol) of this liquid was taken up in 5 ml of diethyl ether under argon, cooled to 0°C with an ice bath, and 2.2 ml of 2 M *n*-propylmagnesium chloride were added. The mixture was stirred at room temperature for 2 hr then quenched with water and NH_4Cl. The aqueous layer was extracted with 20 ml of diethyl ether. The ether extract was dried over anhydrous K_2CO_3, filtered, and the solvent was removed in vacuo. GC-MS analysis of the residue showed the presence of four components (**1a–1d**) with virtually identical mass spectra in a 1 : 1 : 6 : 2 ratio. The GC-FTIR spectra of these compounds are shown in Figures 2 and 3. Typical MS, *m/z* (rel %): 195(M^+, 1), 194(1), 180(5), 153(11), 152(100), 124(5), 82(5), 69(8), 67(8), 55(15). HR-MS, calcd for $C_{13}H_{25}N$, 195.1987; observed for the four GC peaks in order of elution: **1a**, 195.1972; **1b**, 195.1987; **1c**, 195.1973; **1d**, 195.1982.

For derivation from *cis*-piperidine **6**, a sample of *cis*-piperidine **6** purified from the sodium-in-ethanol reduction of **4** was treated in a similar fashion except THF was used in place of diethyl ether as the solvent in the Grignard alkylation. GC-MS analysis of the residue showed the presence of **1a** and **1c** in a 1 : 5 ratio.

For derivation from *trans*-piperidine **7**, a similar treatment of an isolated sample of *trans*-piperidine **7** from the sodium-in-ethanol reduction of **4** provided **1b** and **1d** in a 1 : 2 ratio.

For derivation from *cis*-piperidine **8**, a sample of *cis*-piperidine **8** from the hydrogenation of **5** was treated in a similar fashion except that an excess of 2.0 M methylmagnesium bromide was used in place of *n*-propylmagnesium bromide to give **1a** and **1d** in a 1 : 2 ratio.

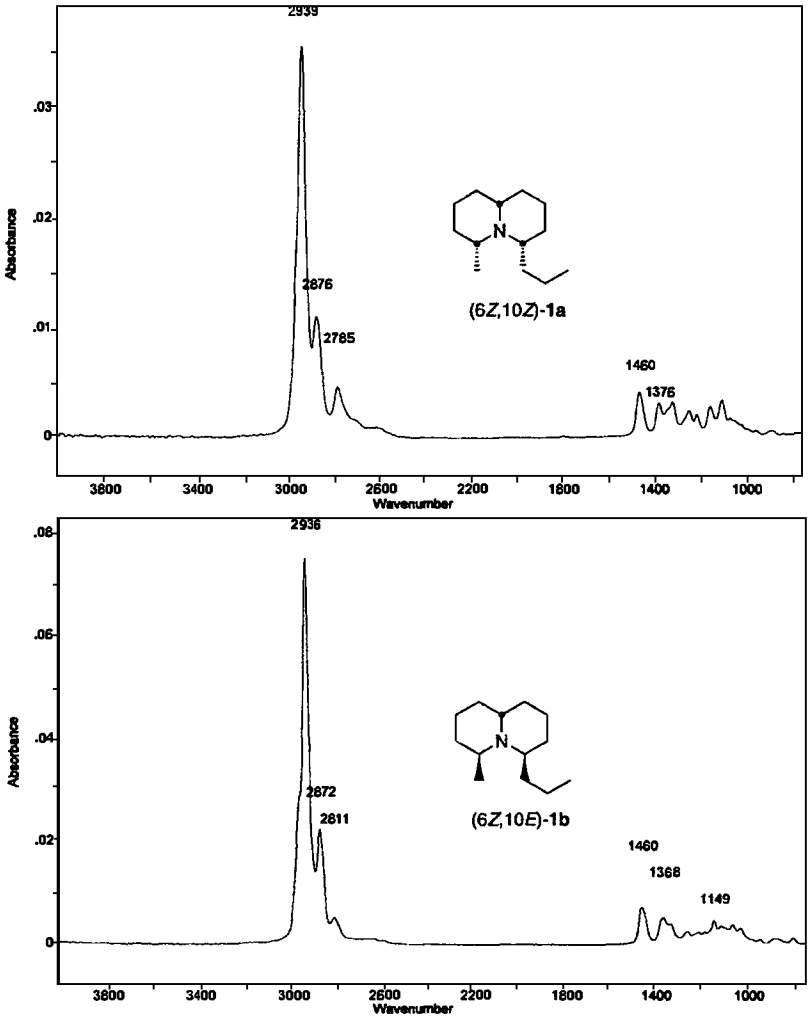


FIG. 2. Vapor-phase FTIR spectra of two synthetic diastereomers of quinolizidine **195C**.

RESULTS AND DISCUSSION

A broad survey of ant extracts is in progress in an effort to identify ant alkaloids that may be dietary sources for some of the over 400 alkaloids that have been characterized in skin extracts from four genera of diurnal, mainly terrestrial dendrobatid frogs of tropical Central and South America; one genus of diurnal,

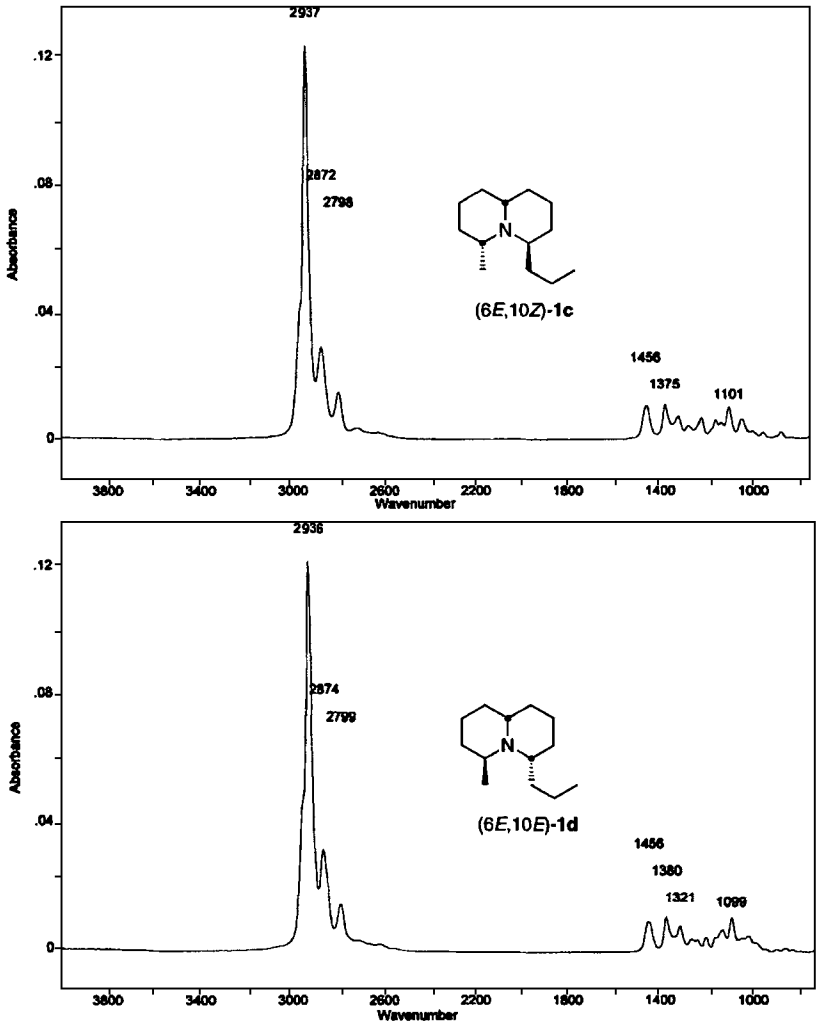


FIG. 3. Vapor phase FTIR spectra of two synthetic diastereomors of quinolizidine **195C**. (see Fig. 2.)

mainly terrestrial mantelline frogs of Madagascar; one genus of nocturnal, terrestrial myobatrachid frogs of Australia; and one genus of diurnal, terrestrial bufonid toads of southeastern South America (Daly et al., 1993; Daly, 1995). Previously, several ant alkaloids of four structural classes had been detected in skin extracts of frogs/toads (Table 1). Representatives of two further structural

TABLE 1. ANT ALKALOIDS DETECTED IN SKIN EXTRACTS OF ALKALOID-CONTAINING FROGS/TOADS[a]

Ant alkaloid	Occurrence		Code name (frogs)
	Ants	Frogs	
Pyrrolidines			
trans-2-Butyl-5-pentyl	*Monomorium pharaonis, Megalomyrmex goeldi, Solenopsis (Diplorhoptrum) punctaticeps*	*Dendrobates* species[b]	**197B**
trans-2-Butyl-5-heptyl	*Monomorium indicum, Megalomyrmex foreli, M. leoninus, Solenopsis (Diplorhoptrum) fugax, Solenopsis (Diplorhoptrum) punctaticeps*	*Dendrobates pumilio, D. granuliferus, D. histrionicus*	**225C**
2-Hexyl-5-pentyl	*Monomorium indicum, Megalomyrmex goeldi, Solenopsis (Diplorhoptrum) molesta*	*Dendrobates pumilio, D. granuliferus*	**225H**
Piperidines (*cis* and *trans*)			
2-Methyl-6-(*Z*-4-nonenyl)	*Solenopsis (Solenopsis)* sp.[b]	*Dendrobates auratus, D. lehmanni*	**223K**
2-Methyl-6-nonyl	*Solenopsis (Solenopsis)* species[b]	*Dendrobates pumilio, D. lehmanni*	**225I**
2-Methyl-6-undecyl	*Solenopsis (Solenopsis)* species[b] *Solenopsis (Diplorhoptrum)* species[b]	*Dendrobates pumilio*	**253J**
Pyrrolizidines			
3-Heptyl-5-methyl	*Solenopsis (Diplorhoptrum)* sp.[b]	*Dendrobates* species,[b] *Phyllobates lugubris, Melanophryniscus stelzneri, Mantella betsileo*	**223H** (two isomers)
(5*E*,8*E*)-3-Butyl-5-hexyl	*Megalomyrmex modestus*	*Dendrobates* species,[b] *Epipedobates bassleri*	**251K** (two isomers)
Indolizidines			
(5Z,9Z)-3-Ethyl-5-methyl	*Solenopsis (Diplorhoptrum) conjurata,*	*Dendrobates pumilio*	**167E**
(5Z,9Z)-3-Butyl-5-methyl	*Monomorium pharaonis*	*Dendrobates* species,[b] *Phyllobates bicolor, P. terribilis, Melanophryniscus stelzneri*	**195B**[c]

(5*E*,9*E*)- and (5*Z*,9*Z*)-3-Butyl-5-propyl	*Solenopsis (Diplorhoptrum)* sp. *molesta* group	*Dendrobates* species,[b] *Phyllobates aurotaenia*, *Melanophryniscus stelzneri*, *Mantella viridis*	**223AB** (5*E*,9*E* and 5*Z*,9*Z*)
(5*E*,9*E*) and (5*Z*,9*Z*)-3-Hexyl-5-methyl	*Solenopsis (Diplorhoptrum) molesta validuiscula*	*Dendrobates auratus*	**223R**
Quinolizidines			
(6*Z*,10*E*)-4-Methyl-6-propyl (1b)	*Solenopsis (Diplorhoptrum)* sp. *picea* group	*Dendrobates* species,[b] *Epipedobates bassleri*, *E. tricolor*, *Minyobates steyermarki*, *Mantella betsileo*, *M. pulchra*, *M. laevigata*, *M. expectata*	**195C**
Decahydroquinolines			
cis-5-Methyl-2-propyl (**3**)	*Solenopsis (Diplorhoptrum)* sp. *picea* group	*Dendrobates* species,[b] *Epipedobates bassleri*, *Minyobates minutus*, *Phyllobates lugubris*, *Mantella betsileo*, *M. laevigata*, *M. pulchra*, *M. crocea*	*cis*-**195A**
cis-2-Methyl-5-propyl (**2**)	*Solenopsis (Diplorhoptrum)* sp. *picea* gr.	*Mantella betsilio*	*cis*-**195J**
cis-2,5-Dipent-4-enyl	*Solenopsis (Diplorhoptrum) azteca*	*Dendrobates pumilio*, *D. granuliferus*, *D. auratus*, *Melanophryniscus stelzneri*	*cis*-**275B**[c]

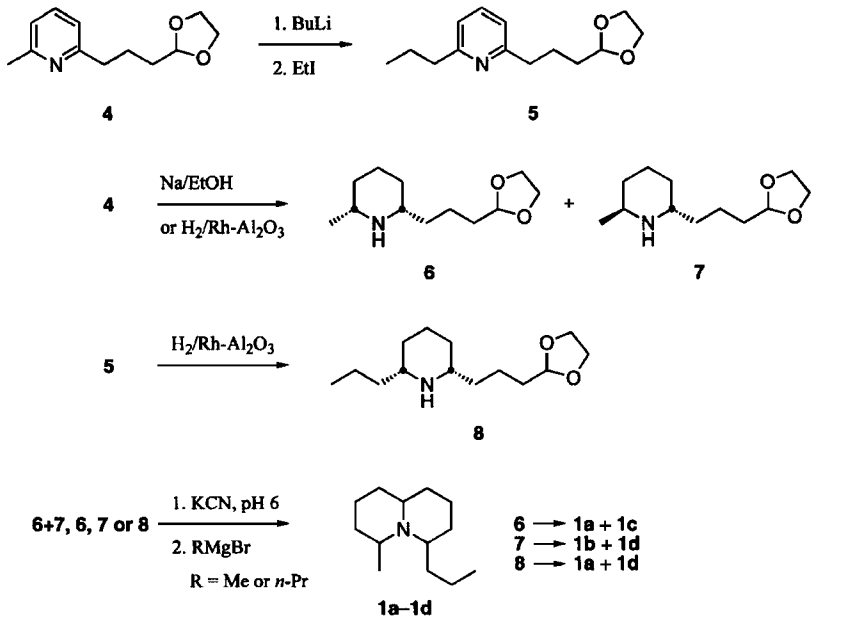
[a]Data from Daly *et al.* (1987, 1996); Jones and Blum (1983); Jones *et al.* (1996); Spande *et al.* (1998 and unpublished).

[b]When four or more species of *Dendrobates* exhibit an alkaloid, it is indicated by "*Dendrobates* species"; multiple species of an ant genus are indicated by "species" following the genus; an undescribed or uncertain ant species is indicated by "sp."

[c]In some cases, different diastereomers of an alkaloid may occur in ant compared to frog, as for example **195B**, where the 5*E*,9*E* diastereomer is the major isomer in dendrobatid frogs, while only the 5*Z*,9*Z* isomer (monomorine I) occurs in ants and for *cis*-**275B**, which occurs in frogs/toads, while 2,5-di-*epi*-*cis*-**275B** occurs in ants.

classes of alkaloids, now discovered in ants and shared with frogs, are also presented in Table 1. These alkaloids are relatively simple and all can potentially originate from a linear carbon-chain precursor.

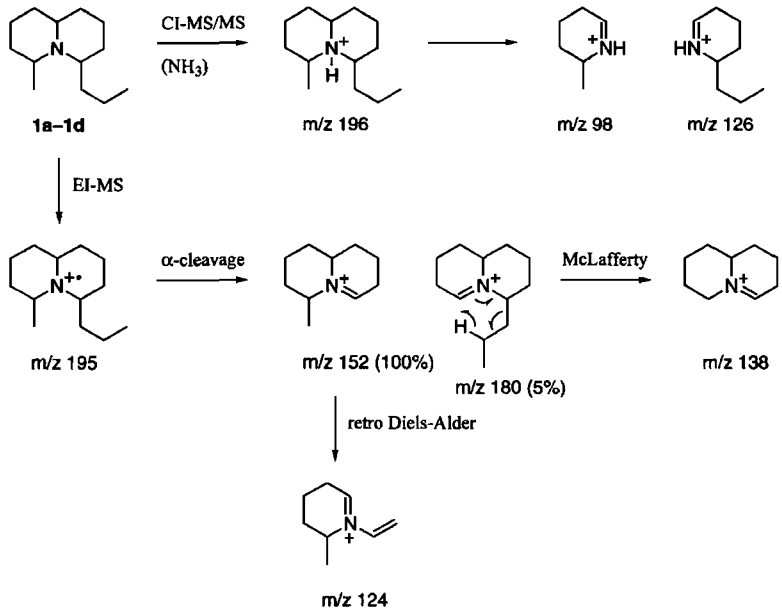
Three isomeric alkaloids with molecular weight 195 ($C_{13}H_{25}N$) were detected by GC-MS in an extract of a Brazilian myrmicine ant, *Solenopsis (Diplorhoptrum)* sp. *picea* group. They occurred in a ratio of about 3 : 1 : 1. The last-eluting alkaloid on GC was identified by its mass spectral fragmentation pattern, FTIR spectrum, and cochromatography with an authentic sample as a *cis*-fused 5-methyl-2-propyldecahydroquinoline (**3**), designated *cis*-**195A**. This was previously detected in skin extracts from dendrobatid frogs (Daly et al., 1993) and skin extracts from four species of mantelline frogs (Daly et al., 1996). The second-eluting alkaloid was also a decahydroquinoline, based on the mass spectral fragmentation, FTIR spectrum, and the presence of an exchangeable hydrogen. This decahydroquinoline had the same Bohlmann band pattern in its FTIR spectrum as *cis*-**195A**, indicating that the H(2) and H(8a) hydrogens were *cis* (Garraffo et al., 1994) and had absorption peaks in the fingerprint region that are diagnostic for *cis*-fused decahydroquinolines, i.e., H(4a) and H(8a) were *cis* (Tokuyama et al., 1991; Garraffo et al., 1994). Thus, the relative stereochemistry at C(2), C(4a), and C(8a) are considered to be the same as in *cis*-**195A**. The relative stereochemistry at C(5) is undetermined. [A 5-*epi*-*cis*-**195A** structure was excluded based on direct comparison with synthetic material provided by Grieco and Parker (1988).] The mass spectral fragmentation shows a substantially larger loss of methyl (32 vs. 5%) in this decahydroquinoline that in *cis*-**195A**, and, thus, it appears to have the methyl and propyl side-chains interchanged so that the favored α-cleavage results in loss of methyl. Cleavage of C_3H_7 from such decahydroquinolines occurs to a significant extent due to loss of carbons 6–8 and can be major, depending on the nature of the C(2) substituent (Yu et al., 1970; Spande et al., 1998). The proposed structure of this decahydroquinoline is *cis*-2-methyl-5-propyldecahydroquinoline (**2**). It has not been previously reported from frog skin and is given the code designation *cis*-**195J**. The predominant alkaloid of the Brazilian ant and the first to elute on GC had no exchangeable hydrogen and showed, on mass spectral fragmentation, a major loss of propyl and a minor loss of methyl, consonant with α-cleavage of such substituents. One weak fragment ion, m/z 124, was perplexing and misleading as it often indicates 3,5-disubstituted indolizidines, which produce this ion from α-cleavage followed by a McLafferty rearrangement (Garraffo et al., 1993b). The EI-MS/MS data clarified the origin of this fragment. The FTIR spectrum had Bohlmann bands weaker than those of disubstituted indolizidines and hence a 4-methyl-6-propylquinolizidine seemed the only reasonable structure. Alkylation of the intermediate cyanopiperidines obtained from **6** and **7** (Grierson et al., 1986) afforded all four possible quinolizidine diastereomers for comparison (Scheme 1). The second of the four eluting diastereomers on GC was identical, with respect to GC



SCHEME 1. Synthesis of the four diastereomers of quinolizidine **195C**.

retention time and mass and infrared spectra, with the ant alkaloid. Mass spectral analysis included the use of chemical ionization tandem mass spectrometry (CI-MS/MS) in which the protonated molecular ion obtained with ammonia as the reagent gas was subjected to MS/MS analysis in a combined quadrupole–ion trap mass spectrometer. In contrast to EI-MS, no radical-induced α-cleavage was seen, but instead, cleavage to protonated even-mass ions comprising one ring and its substituent, represent major fragmentations. Such CI-MS/MS analysis provided rational, predictable spectra (viz. m/z 126 and 98 plus weaker ions, see Scheme 2) and data complementary to those obtained by EI-MS analysis. The technique has proven to be widely applicable for structural assignments to "izidine" and other alkaloids (unpublished results).

Conventional EI-MS/MS studies with quinolizidines **1a–1d** indicated that one α-cleavage ion at m/z 180 (M^+-CH_3) generates a daughter ion at m/z 138 (100%) by a McLafferty rearrangement and weaker ions at m/z 124 and 84, whose intensities depend upon stereochemistry. The other α-cleavage ion, m/z 152 (M^+-C_3H_7), gave a major ion at m/z 124 by a retro Diels-Alder process and a weaker fragment ion at m/z 84, whose intensity, again, was dependent upon stereochemistry. Both m/z 138 and 124 ions, although weak, are observed in EI-MS of **1a–1d**.



SCHEME 2. Proposed mass spectral fragmentations of quinolizidine **195C**.

The ant quinolizidine was recognized by comparison of GC retention times, mass spectra, and FTIR spectra as identical to alkaloid **195C**, previously detected in many frog skin extracts but of unknown structure. Sufficient amounts of alkaloid **195C** were isolated from skin extracts of 19 specimens of the Madagascan mantelline frog *Mantella betsileo* Grandidier 1872 to obtain a ^{1}H NMR spectrum. An overlapping methyl doublet and methyl triplet at δ1.0 and three downfield CHN protons at δ 3.7–4.0 were present and are consonant with a quinolizidine structure having methyl and *n*-propyl substituents at C(4) and C(6). Remarkably, an extract of the population of *Mantella betsileo* from Farakaraina showed, as minor components, the same three alkaloids **1**, **2**, and **3** that had been seen in the ant, but in a slightly differing ratio of 3.5 : 1 : 1.8, respectively. The other two *M. betsileo* populations had **1–3** in similar ratios.

The stereochemistry of the ant and frog quinolizidine **195C** was established as the (6*Z*,10*E*)-4-methyl-6-propylquinolizidine **1b** as follows: When the synthesis commenced with the *cis*-2-methylpiperidine **6** and employed *n*-propylmagnesium chloride in the last step, quinolizidines (**1a** and **1c**), corresponding to the first and third GC peaks, were obtained, while synthesis with *trans*-2-methylpiperidine **7** gave the second- and fourth-eluting GC peaks, i.e., **1b** and **1d**. When the synthesis commenced with the *cis*-2-*n*-propylpiperidine **8** and

employed methylmagnesium bromide in the last step, the first and fourth peaks (**1a** and **1d**) were obtained. Since **1a** arose from both **6** and **8**, it must have both rings *cis* substituted and thus **1d**, uniquely produced from **8**, has the propyl-bearing ring *cis* but the methyl-bearing ring *trans*. Since **1d** also resulted from **7**, the other isomer from that reaction, **1b**, identical to the naturally occurring ant and frog quinolizidine (GC peak 2), must be the diastereomer where both rings are *trans* substituted, i.e., the 6*Z*,10*E*[6] structure. This is consistent with the weak Bohlmann band pattern observed in the GC-FTIR spectrum of **195C**. It seems likely that diastereoisomer **1b** will be in the *cis*-fused quinolizidine ring conformation, since several simple 4-axially substituted quinolizidines have been demonstrated by ^{1}H NMR to be in the *cis*-fused conformation (cf. Matsunaga et al., 1967; Yasuda et al., 1977).

In conclusion, the present study documents the occurrence of two 2,5-disubstituted *cis*-decahydroquinolines and a 4,6-disubstituted quinolizidine in a myrmicine ant. A parallel study has demonstrated two 2,5-dipent-4-enyl decahydroquinolines in alate queens of a Puerto Rican myrmicine ant *Solenopsis (Diplorhoptrum) azteca* (Spande et al., 1998), but in that case the diastereomers found in the ant have not yet been detected in frog skin extracts. The decahydroquinoline *cis*-**195A** occurs in skin extracts from dendrobatid frogs and in mantelline frogs of Madagascar (Table 1). The decahydroquinoline *cis*-**195J** has now been found in one species of a mantelline frog. Quinolizidines have not been previously reported from ants. 1,4-Disubstituted quinolizidines had been previously reported from frogs (Jain et al., 1996), but now it is clear that the frog skin alkaloid **195C**, which occurs in extracts of dendrobatid frogs of Costa Rica, Panama, Colombia, Ecuador, Peru, Venezuela, and Brazil and in mantelline frogs of Madagascar, is a 4,6-disubstituted quinolizidine and is identical to the Brazilian ant quinolizidine. Such 4,6-disubstituted quinolizidines are rare in nature, being limited, as far as we know, to the plant alkaloid porantherilidine (Johns et al., 1974). The occurrence of the triad of two decahydroquinolines and a quinolizidine in a Brazilian ant and the skin of a Madagascan mantelline frog suggests that a closely related Madagascan ant probably is the dietary source for the three frog skin alkaloids. It appears likely that DHQs **2** and **3** arise from ring closures from 13-carbon *cis*-piperidines, perhaps via enamines, while the quinolizidine **1** likely arises from a 13-carbon *trans*-piperidine. Both *cis*- and *trans*-2,6-dialkylpiperidines occur in myrmicine ant venoms (Jones and Blum, 1983; Jones et

[6]In this notation of assigning structures to "izidine" diastereomers, the hydrogens at the positions of the substituents [in this case H(4) and H(6)] and the bridgehead hydrogen H(10) are referred to the orientation of the H at the position of the lowest numbered substituent [in this case H(4)] and are either *cis* (*Z*) or *trans* (*E*) relative to that H. An alternative nomenclature employed α- and β-descriptors for substituents (cf. Johns et al., 1974) but did not specify the configuration for H(10), which was assumed to be that of the usual *trans*-fused ring system. *cis*-Fused quinolizidines, although uncommon, also exist.

al., 1990). Many such ants are known to be prey items for dendrobatid frogs, particularly those of the genus *Dendrobates* (Silverstone, 1975, 1976). They could serve as dietary sources for the alkaloids that dendrobatid and mantelline and probably myobatrachid and bufonid frogs/toads sequester unchanged into skin glands for use in chemical defense against predators.

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Brief Communication

PROBLEMS ENCOUNTERED IN FEEDING MICROENCAPSULATED OILS TO A FOLIVOROUS MARSUPIAL, THE COMMON RINGTAIL POSSUM (*Pseudocheirus peregrinus*)

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Abstract—In our study of interactions between marsupial folivores and *Eucalyptus*, we have examined the role of terpenes, including the use of bioassay experiments where terpenes are added to an artificial diet. In pursuit of greater ecological realism in these experiments, we have sought means of packaging terpenes more realistically, by using microencapsulation to simulate oil glands of leaves. We report here on a preliminary experiment with microencapsulated olive oil (intended for use as an experimental control) in which the food intake of the animals was substantially reduced, to the point that starvation appeared imminent and we aborted the experiment. We discuss why this occurred and recommend caution to others intending to use microencapsulated terpenes (or other oils) in herbivore diets.

Key Words—Microencapsulation, terpene, plant–herbivore interaction, possum, *Eucalyptus*.

INTRODUCTION

Terpenes are volatile plant secondary metabolites that have been the subject of a number of studies of plant-herbivore interactions (Foley et al., 1987;

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Edwards *et al.*, 1993; Hume & Esson, 1993; Duncan et al., 1994; Lawler & Foley, 1999). Because they are found in varying concentrations between and within species, have distinct tastes and odors, and are toxic to some animals in small doses (McLean & Foley, 1997), they are often suspected to be deterrents to feeding by both mammalian and insect folivores. Several studies have investigated their effect on mammalian feeding by using correlative analysis of the relationship between food intake and concentration of terpenes in the foliage (Hume and Esson, 1993; Lawler et al., 1998a). However, such correlative studies do not show cause and effect, and bioassay studies, in which isolated terpenes are added to artificial diets, are required to ascribe a definite role to the terpenes (Lawler et al., 1998a).

The presentation of terpenes in artificial diets in an ecologically realistic manner is problematic. In plants, terpenes in the foliage typically are contained in microscopic glands. In contrast, most bioassay experiments with terpenes are conducted with the liquid terpenes added freely to the diet or adsorbed onto a dry component of the diet (Reichardt et al., 1990; Lawler et al., 1998a, 1999). Because terpenes are highly volatile and odoriferous, this presentation results in an artificial diet that presents much stronger taste and olfactory cues to the animal, even thought the total terpene concentration may be similar to that in foliage. If these aspects are important (Lawler et al., 1999), then results obtained with artificial diets may not be comparable to those obtained with foliage diets. Stronger signals would likely exaggerate the importance of terpenes in artificial diets. As a consequence, we have sought means of packaging terpenes for addition to diets in a realistic manner, one of which has been microencapsulation (Clancy et al., 1992). The microencapsulation technique used was coacervation of gelatin and acacia resin in an emulsion of the target oil in water. Manipulation of the pH causes the gelatin and acacia to bind at the oil–water interface of each oil droplet to form the capsule. The technique has been widely used in a variety of applications, such as feeding of marine larvae (Southgate and Lou, 1995), as well in testing, similar to ours, on the effects of terpenes on insect feeding (Clancy et al., 1992). The size of the capsules can be controlled by the speed at which the emulsion is blended, producing capsules of sizes similar to the oil glands found in leaves.

METHODS AND MATERIALS

Preparation of Capsules. Several attempts were made to produce the capsules in our laboratory by using the technique described in Southgate and Lou (1995). We were unsuccessful, as terpenes are much less viscous than the oils commonly used in this procedure. Further, we required the capsules to be in powder form for addition to the diet, which required hardening of the capsules

with glutaraldehyde (Clancy et al., 1992) before drying. Finally, we contracted a commercial manufacturer to produce capsules containing a terpene (1,8-cineole) and a separate batch containing olive oil, intended for use as experimental controls (as we had previously established that addition of free olive oil to the diet had no effect on food intake). The capsules that were supplied were approximately 12.5% oil by weight.

Diet Composition. (Lawler et al., 1998a), established that a threshold concentration of cineole of approximately 2.6% of the dry matter (DM) of the diet was required to produce any reduction in food intake by common ringtail possums. We decided to begin experiments with a concentration (approximately 8% DM) that would normally decrease intake to approximately 40% of normal intake, in order for differences between intakes of free and encapsulated diets to be statistically discernible. A preliminary experiment was conducted to test the control capsules (with olive oil) before commencing a full experiment comparing the effects of free and encapsulated cineole. However, we found that the diet had to be substantially modified to make it palatable to the animals. The capsules were hydrophobic and did not mix well with the diet, requiring the addition of substantially more fruit to make it cohesive and palatable to the animals. The final diet contained approximately 60% capsules (DM) in order to reach an olive oil concentration of 8% (DM) (equivalent to that desired for terpenes in the subsequent experiments). Due to the failure of this initial experiment (see Results), it was considered unsafe to continue with further experiments.

RESULTS

All of the 12 adult ringtail possums offered the diet with microcapsules containing olive oil on the first night showed an unexpected reduction in food intake (Figure 1). Only two were given the capsule-containing diet again on the second night while the remainder were returned to the normal basal diet. Again, the intakes were low. The two animals given capsules for a second night had extremely low intakes and, surprisingly, those not given capsules (which had eaten them the previous night) reduced intakes even further than they had when given the capsules. At this point the experiment was discontinued and after another night of similarly low intakes, we began to offer the animals several alternative foods to induce them to feed. These included the basal diet, highly palatable foliage from two species of *Eucalyptus*, and honeyed water. Intakes gradually recovered, and the animals were again weaned off honeyed water and foliage and back onto the artificial diet. It was not until 12 days after first giving the animals the microcapsules that intakes of all individuals had reached levels similar to those recorded previously.

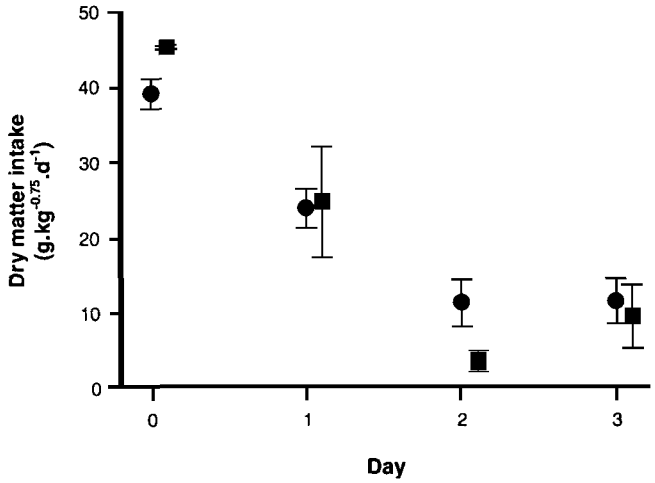

FIG 1. The effect of microcapsules containing olive oil on food intake by common ringtail possums. Day 0 represents the day before the experiment when animals were given the basal diet only. Circles represent animals given microcapsules on day 1 only and basal diet on subsequent days. Squares represent animals given microcapsules on days 1 and 2. Values are means ± standard error.

DISCUSSION

The use of microcapsules to present terpenes to folivorous marsupials in artificial diets in an ecologically realistic fashion is theoretically sound. However, the data presented here show that in practice the technique is more complex. This study was conducted as part of a larger project studying the effect of deterrent compounds on the interaction between marsupial folivores and *Eucalyptus*. It is now becoming clear that a specific group of compounds other than terpenes is the major determinant of deterrency (Lawler et al., 1998a, 1999), and hence the work required to investigate fully the problems encountered here is not warranted. However, terpenes have been suggested to be important in a number of other interactions, such as between deer and spruce (Duncan et al., 1994) and between leaf-chewing insects and *Eucalyptus* (Edwards et al., 1993). It is likely that others will attempt to adopt microencapsulation in their studies, and hence this note is written as a caution. Below we discuss possible reasons for the problems encountered.

Perhaps the most significant factors in determining if this will be a viable technique are the ratio of oil (or terpene) to capsule wall (payload) and the concentration required in the diet to produce the desired effect. In their study of the effects of individual terpenes on performance of insect herbivores of Douglas fir

(*Pseudotsuga menziesii*), Clancy et al. (1992) had similar payloads of terpenes but required dry matter concentrations of not more than 0.8% and generally less than 0.4%. In contrast, we required a minimum of 7% dry matter, some 10 times higher. Microencapsulation may be a more viable technique for dietary studies in those species with lower thresholds for the encapsulated compound. It is now known that ringtail possums can tolerate dietary terpenes at levels higher than those found in most resistant *Eucalyptus* foliage (Lawler et al., 1999), and hence the use of microencapsulated diets will not be viable unless the terpene payload can be substantially increased. It should be noted, however, that others have used microencapsulated oils (corn oil) in mammalian diets at high concentrations (approximately 3% of dry matter) without encountering such problems (M. D. Dearing, personal communication).

Given that similar amounts of microcapsules of similar composition have been fed successfully to other mammals, the effect of these capsules on intake of common ringtail possums is difficult to explain. The effect of the capsules on the nitrogen content of the diet is an unlikely explanation, because the diet with capsules was 1.7% N (dry matter) (basal diet was 1.9% N), while common ringtail possums can easily maintain themselves on diets substantially lower in nitrogen. It is also unlikely that the olive oil itself caused the effects seen, as similar doses (7.5% DM) added freely to the diet had no such effect on intake (Lawler, unpublished data).

We consider it likely that the particular digestive physiology of common ringtail possums is a factor in the problems encountered here. Ringtail possums cope with a fibrous diet by selective retention of fine particles in the cecum where fermentative digestion takes place (Cork and Foley, 1991). The capsules are of appropriate size to be retained, so one explanation may be that the capsules have allowed the oil to be carried through to the cecum, rather than absorbed earlier as is usually the case. This may, in turn, result in disruption of fermentation, perhaps through effects on the gut flora. If capsules are not ruptured or broken down previously, then perhaps the olive oil is released into the cecum, or it may be a result of fermentation of the capsule wall material itself, if capsules are previously ruptured in the stomach. Animals often become sick if the site of digestion of diet components changes. McLean et al. (1995) found that when ringtail possums were dosed by gastric lavage with citronellal, they showed no ill effect, but when the same compound was dosed in peanut oil, animals stopped feeding for four to five days, and one died. In that case, the peanut oil may have protected the citronellal from acid rearrangements in the stomach, causing unchanged citronellal to be absorbed from the small intestine. Cheeke and Patton (1980) also concluded that similar symptoms shown in rabbits eating grain-rich diets were due to the soluble carbohydrates being digested in the cecum and not the small intestine.

Unfortunately, it is not possible to assess the likely effects of the capsule

material itself as proprietary arrangements with the supplying company prevent our revealing the detailed nature of the capsules. Capsules produced by coacervation are frequently used in animal feeds for a wide variety of taxa (e.g. Clancy et al., 1992; Southgate and Lou, 1995), and the capsules used here were approved by the United States Food and Drug Agency for human consumption. Hence, we assumed they would be safe for this purpose, but cannot eliminate the possibility that the unforseen problems are a result of the much higher concentrations given to these animals, relative to any human application.

In conclusion, for anyone who intends to use this technique for a similar purpose, there are several problems to address. Firstly, it is important to determine both the active dose of the compound required and the achievable payload, so that some assessment can be made of whether the diet's appearance and palatability will be fundamentally changed in order to reach this concentration. Secondly, it is important to consider the likely effect of the capsules themselves, to prevent or allow for the problems encountered here. Finally, the question of cost is significant. In this instance, the capsules cost over US$3000 and required the use of over five times the amount of terpene required to be encapsulated. This cost will vary depending on the physical and chemical properties of the compound to be encapsulated, but may in many cases prove to be the limiting factor.

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Erratum

"Sex pheromone of female vine bud moth, *Theresimima ampellophaga* comprises (2*S*)-butyl (7*Z*)-tetradecenoate," Subchev et al. *J. Chem. Ecol.* 24: 1141–1151.

Due to a mistake in labeling synthetic samples, the sex pheromone of the female vine bud moth, *Theresimima ampellophaga*, was erroneously assigned to show the (2*S*)- configuration. The correct structure is (2*R*)-butyl (7*Z*)-tetradecenoate. Consequently, in the title of the paper, the Abstract, Key Words, legends to Fig. 1, Fig. 2, and Table 1, as well as on p. 1146 (line 14 from bottom), p. 1148 (lines 3, 8, and 9 from bottom), and p. 1149 (line 4 from bottom), (2*S*) must be replaced by (2*R*). On p. 1148 (line 11 from bottom), (2*R*) must be replaced by (2*S*). Rotation values given on p. 1146 are correct, i.e., the synthetic sample showing the natural pheromone structure has a value of $[\alpha]_D^{21} = -9.60$. In contrast to earlier findings, a recently investigated sample of the natural pheromone proved to be stereochemically less pure, showing an *R*–*S* ratio of 9 : 1.

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ROLE OF DETOXIFICATION OF PLANT SECONDARY COMPOUNDS ON DIET BREADTH IN A MAMMALIAN HERBIVORE, *Trichosurus vulpecula*

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Abstract—Theory predicts that mammalian herbivores detoxify different classes of plant secondary compounds via separate metabolic pathways and that generalist herbivores maintain broad diet breadth to avoid overloading individual detoxification pathways. We tested the hypothesis that a generalist marsupial herbivore, the common brushtail possum, *Trichosurus vulpecula*, can maintain a higher intake of food when allowed to select from two diets containing different profiles of secondary compounds (phenolics and terpenes) than when given access to the diets individually. Diets consisted of a fruit and vegetable mash to which was added ground leaves of either *Eucalyptus melliodora* or *E. radiata*. *E. melliodora* and *E. radiata* differ in their concentrations and types of secondary compounds. Brushtail possums include these eucalypt species as part of their natural diet. We measured food consumption and detoxification metabolites of possums on these diets. Consistent with the hypothesis, animals presented with a choice of both diets consumed more food than animals given diets singly. One of the two indicators of detoxification, acid load in urine, differed significantly between diets while the other, glucuronic acid, did not. These results provide partial support for the hypothesis that diet breadth is governed by detoxification abilities.

Key Words—Diet breadth, herbivory, phenolics, terpenes, *Trichosurus vulpecula*, detoxification.

INTRODUCTION

In their seminal paper on feeding strategies of herbivorous mammals, Freeland and Janzen (1974) proposed that the majority of mammalian herbivores were

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obligate dietary generalists due to limitations of their detoxification systems. Mammals possess several distinct detoxification pathways that individually process particular classes of compounds, although some redundancy exists (Sipes and Gandolfi, 1986). Freeland and Janzen (1974) predicted that the large quantities of similar plant secondary metabolites ingested from a diet of a single species of plant should exceed the capacities of the one or two detoxification pathways specific to those compounds. They predicted that mammalian herbivores could circumvent overloading any one pathway by feeding on a wide variety of plants, each containing different types of secondary metabolites that would be processed through distinct detoxification pathways.

In the 25 years since Freeland and Janzen's (1974) paper, many of the secondary metabolites occurring in plants (e.g., Adams et al., 1981; Bryant et al., 1983; Reichardt et al., 1990; Sinclair et al., 1988) as well as enzymes of the mammalian detoxification system have been described at the molecular level (e.g., Dutton, 1980; Jakoby, 1980; Sipes and Gandolfi, 1986; Testa and Jenner, 1981). Myriad studies conducted on the diets of herbivorous mammals support the assumption that most mammalian herbivores are generalist feeders (e.g., Cameron and Rainey, 1972; Dearing, 1996; Dial, 1988; Freeland, 1991; Ganzhorn, 1988; Kerle, 1984). In laboratory studies, mammalian herbivores select a diet of several plant species even when presented with ample quantities of their most preferred food types (Atsatt and Ingram, 1983; Freeland and Winter, 1975; Dearing unpublished data). Moreover, food intake is enhanced when they are presented with more than one type of food (Freeland and Winter, 1975). These lines of evidence suggest that the generalist feeding behavior of mammalian herbivores is imposed by limitations of the detoxification system; however, this hypothesis has not been tested experimentally.

An alternative hypothesis is that no single species of plant can provide all of the necessary nutrient requirements, and thus, mammals must consume a number of species to obtain a nutritionally adequate diet (Westoby, 1978). Whereas gross energy content does not vary greatly among many plant species, other variables, e.g., fiber and nitrogen, may vary tremendously between and within species as well as between different plant tissue types (Dearing and Schall, 1992). Mammals have demonstrated abilities to discriminate differences in such variables. For example, the eastern woodrat, *Neotoma floridana*, discriminates between diets that are 17.5% and 14% protein, preferring the former (Post, 1993).

We tested Freeland and Janzen's (1974) hypothesis of detoxification limitation by using a wild herbivore fed natural foods and monitoring fluxes through detoxification pathways. The system consisted of the brushtail possum, *Trichosurus vulpecula*, and two species of *Eucalyptus*, *E. melliodora* and *E. radiata*. Brushtails are common to the forests of eastern Australia and are typical generalist herbivores in that individual possums select a wide range of plant species

for their diets. In many habitats, *Eucalyptus* species comprise significant portions of their diets (Freeland and Winter, 1975; Kerle, 1984).

The two eucalypt species used in the experiment produce different types of secondary chemicals that are potentially processed through different detoxification pathways. *E. radiata* is noted for its high levels of terpene oils, which in some chemotypes approach 8% of the dry mass (Foley, 1992). Based on data in the literature, we predicted that the compounds in *E. radiata* would be detoxified primarily through the phase 1 pathway (also known as functionalization). In the phase 1 pathway, xenobiotics are made more hydrophilic and polar through oxidation, reduction, or hydrolysis, and hence easier to excrete (Sipes and Gandolfi, 1986). Many terpenes have been shown to be oxidized after ingestion (McLean et al., 1993; Southwell et al., 1980). Because the metabolites produced from the phase 1 pathway are typically unconjugated organic acids (McLean et al., 1993), urine pH and titratable acids in the urine can be used as indicators of detoxification (Foley and Hume, 1987; Foley et al., 1995).

E. melliodora also has terpenes; however, they typically occur in lower concentrations (1–2% of dry mass) with cineole and α-phellandrene being the dominant components (Edwards et al., 1993). *E. melliodora* is reported to contain significant levels of phenolics, particularly condensed tannins (Foley and Hume, 1987). Although absorption of condensed tannins by the gut is controversial, some data suggest that condensed tannins are absorbed and require detoxification (Clausen et al., 1990; Distel and Provenza, 1991; Harmand and Blanquet, 1978). The phenolics of *E. melliodora* have not been examined in as much molecular detail as have terpenes; thus, our investigation of them was restricted to the class level. Many phenolics appear to be detoxified through phase 2 pathways (Sipes and Gandolfi, 1986). In phase 2 pathways, xenobiotics are conjugated with endogenous molecules that are hydrophilic. The primary phase 2 pathway employed by marsupials is the glucuronic acid pathway (Baudinette et al., 1980).

Although *E. radiata* and *E. melliodora* differ in their profiles of secondary compounds, they are reported to have similar nitrogen, energy, and fiber contents (Foley and Hume, 1987). This similarity allowed us to test the hypothesis of detoxification limitation while holding nutrient levels relatively constant.

Using this study system, we tested the following predictions generated from the detoxification-limitation hypothesis: (1) food intake should be greater when possums are given both *Eucalyptus* diets simultaneously than when they are given either diet individually; (2) the types or amounts of detoxification products excreted by possums should differ between *Eucalyptus* treatments; (3) when feeding on both diets simultaneously, concentrations of detoxification products excreted in the urine should be intermediate to those produced when animals are feeding on diets individually.

Here we provide the first experimental evidence that supports the hypothesis that limitations of the detoxification system impose a generalist feeding strategy on mammalian herbivores.

METHODS AND MATERIALS

Collection and Treatment of Leaf Material

We collected mature leaves from several trees (5–7 individuals per species) of *Eucalyptus radiata* and *E. melliodora* from forests near Tidbinbillia, Australian Capital Territory, Australia. Leaves were collected simultaneously to minimize any possible seasonal differences in chemistry. They were stored in a freezer for a week prior to being freeze-dried. Freeze-dried leaves of each species were ground in a Wiley mill through a 1-mm mesh and homogenized. They were analyzed colorimetrically in duplicate for total phenolics (Singleton and Rossi, 1965) and condensed tannins (HCl–butanol method) (Porter et al., 1986). The standards used in these assays were tannic acid for the total phenolics assay and quebracho for the condensed tannins assay. Terpenes were identified and quantified by gas chromatography with tridecane as an internal standard (Edwards et al., 1993). To verify that the leaves used did not differ in nutritional content, we measured total nitrogen (Kjeldahl assay) and fiber (acid detergent analysis) (Goering and Van Soest, 1970) in duplicate.

Animals

Six adult possums were trapped from woodlands on the property of the CSIRO's Division of Wildlife and Ecology in Canberra, Australia. Because many of the females had pouch or back young, we restricted our study to males. Possums ranged in weight from 2.44 to 3.38 kg ($x = 2.77$, SE = 0.12 kg), and they were kept in wire cages (1 m × 1 m × 1 m) with a nest box and wire mesh bottoms. Water was provided ad libitum.

Diets

Possums were fed a maintenance diet of dry ground alfalfa (*Medicago sativa*), fresh *Beta vulgaris* cicla (Swiss chard or silver beet), carrots, apples, sugar, and ground *Eucalyptus* leaves (Table 1). *Eucalyptus* leaves were included in the maintenance diet so that possums would maintain the same level of inducible detoxification enzymes prior to and between treatments. All diet components were finely chopped in a food processor to produce particle sizes small enough to prevent selective foraging. Possums maintained weight on the maintenance diet.

Treatment diets consisted of the maintenance diet with alfalfa, Swiss chard,

TABLE 1. COMPONENTS OF MAINTENANCE AND TREATMENT DIETS[a]

Maintenance diet	Treatment diet
35% apple	34% apple
6% carrot	6% carrot
10% sugar	10% sugar
25% alfalfa	50% *Eucalyptus* sp.
14% *B. vulgaris*	
5% *E. melliodora*	
5% *E. radiata*	

[a]All components are expressed on a dry matter basis. For the treatment diets, the *Eucalyptus* added was either *E. radiata* or *E. melliodora*.

and *Eucalyptus* components replaced with freeze-dried ground leaves of either *E. radiata* or *E. melliodora*. We chose this approach over that of feeding fresh leaves because of the enormous heterogeneity that may occur among leaves both within and among trees. We conducted preliminary feeding trials to determine which concentrations of *Eucalyptus* leaves would depress food intake to significantly less than maintenance without being completely deterrent. This tactic was necessary to ensure that when possums were presented with both treatments, they would eat from both and not only their preferred treatment. Fifty percent of each treatment diet (dry weight) consisted of either *Eucalyptus radiata* or *E. melliodora*. Each possum received all of the following treatments: *E. radiata* diet only (405 g wet weight/day), *E. melliodora* diet only (405 g wet weight/day), and a combination of both (405 g/day of each treatment). The quantity of the diet treatments offered singly, i.e., 405 g, was in excess of what possums require to maintain weight and was approximately 2× more than possums voluntarily consumed during preliminary trials. For the combination treatment, *E. radiata* and *E. melliodora* were presented in separate dishes placed side by side. This presentation allowed possums to select the amount of each treatment. Treatments were fed to each animal for five consecutive days followed by five days on the maintenance diet prior to the next treatment. Diets were prepared fresh each day. Leftovers were removed daily and dried before weighing. To account for possible effects of diet sequence, we used a crossover design where different animals received the various treatments in different orders.

Data Collection and Treatment

Animals were weighed at the start and end of each treatment. Food intake, urine volume, total glucuronic acid production (Blumenkrantz and Asboe-Hansen, 1973), and urine pH were measured daily. Urine was collected at room temperature in 500-ml flasks under each cage. Urine pH was measured with a

pH meter on the initial sample collected at 24°C. Titratable acids in the urine were measured during the last two days of each treatment. Twenty milliliters of urine at 24°C were titrated to pH 6.6 with 0.2 N KOH.

We compared the percent change in body mass from the start of each treatment to the end of each treatment by using repeated measures analysis of variance (ANOVA) and Tukey's HSD for differences among diets. To compare the effects of diet treatment on food intake, urine pH, titratable acid, and glucuronic acid concentration, we averaged data from the last two days of the treatment for each possum. We used this approach to remove the effects of acclimation to the diet, which may have occurred during the initial days and to allow for maximal induction of detoxification enzymes (Sipes and Gandolfi, 1986). Results were analyzed by repeated measures ANOVAs and Tukey's HSD for differences between diets. Titratable acid and glucuronic acid concentrations were not normally distributed and were transformed (square root-arcsin and natural log, respectively) prior to analysis. We performed correlations on amount consumed of diets fed individually, indicators of detoxification (glucuronic acid and pH), and number of days on each diet to identify potential relationships among these factors.

RESULTS

There was little difference in the nutritional contents of *E. melliodora* and *E. radiata*. Both had similar concentrations of fiber (26.0% vs. 24.1% res. (Acid Detergent Fiber) ADF, $P > 0.05$, $N = 4$). *E. radiata* had slightly but significantly higher levels of nitrogen compared to *E. melliodora* (1.5% vs. 1.39%, $P = 0.05$, $N = 4$).

E. radiata and *E. melliodora* differed in their secondary compound profiles. *E. radiata* contained significantly higher levels of total phenolics (240.5 ± 5.8 vs 188.8 ± 8.5 mg/g tannic acid equivalent, $P < 0.05$, $N = 4$) and equal levels of condensed tannins compared to *E. melliodora* (354 ± 11 vs. 347 ± 0.4 mg/g quebracho equivalents, $P > 0.05$, $N = 4$). *E. radiata* had higher total concentrations of terpenes compared to *E. melliodora* (2.08% vs. 1.28% oil/dry mass, respectively). The prominent terpenes in *E. radiata* were sesquiterpenes and oxygenated monoterpenes, whereas cineole comprised the majority of the terpene spectrum of *E. melliodora* (Figure 1). Statistical comparisons of terpene content are unavailable as only one pooled sample of each species was assayed for terpenes.

Possums lost weight during all diet treatments (Table 2). The greatest change in body mass occurred when they were feeding on the *E. radiata*-only treatment; there was no significant difference in weight loss of possums feeding on *E. melliodora*-only and both diets (Table 2). Possums recovered all weight lost on treatments during the five-day rest period.

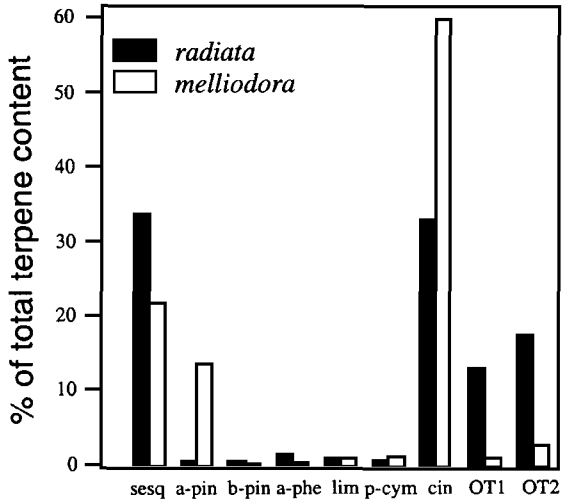

FIG. 1. Secondary chemistry of *Eucalyptus melliodora* and *E. radiata*. Terpene abbreviations are: sesq, sesquiterpenes; a-pin, α-pinene; b-pin, β-pinene; a-phe, α-phellandrene; lim, limonene; p-cym, paracymene; cin, cineole; OT1, uncharacterized oxygenated terpene 1; OT2, uncharacterized oxygenated terpene 2.

The amount of food consumed varied significantly with treatment (Figure 2, Table 3). Possums consumed 33% more food when offered both diets simultaneously compared to either diet offered singly. When offered both diets simultaneously, possums ate significantly less of each diet than when presented singly. However, this difference was statistically significant for only the *E. radiata* treatment (*E. radiata:* $t = 3.15$, $df = 5$, $P = 0.025$; *E. melliodora:* $t = 2.21$ $df = 5$, $P = 0.07$). In none of the treatments did possums achieve food intake levels comparable to that of the maintenance diet, approximately 40 g dry matter/kg$^{0.75}$/day. Regardless of treatment, they never consumed all of any one treatment offered in a 24-hr period, i.e., leftovers were always present.

TABLE 2. MEAN PERCENT WEIGHT CHANGE OF POSSUMS AFTER EACH DIET TREATMENT[a]

Diet treatment	Weight change
E. melliodora only	−4.4 (0.7) a
E. radiata only	−6.5 (1.1) b
Both	−3.1 (0.6) a

[a]Standard errors are given in parentheses. Changes in weight with different letters indicate significant differences (Tukey's HSD).

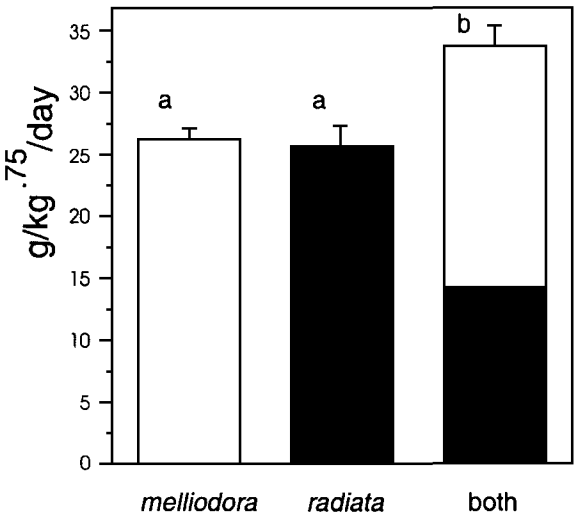

FIG. 2. Average food intake (+ 1 SE) in grams for the last two days on each diet treatment. Intake was standardized for metabolic body mass. Intakes with different superscripts are significantly different. The open segment of "both" represents the amount of *E. melliodora* consumed when both diets were presented simultaneously, the filled segment represents the amount of *E. radiata* consumed.

TABLE 3. SUMMARY OF REPEATED MEASURE ANOVAS[a]

	F ratio	*P*
% Change mass		
Within subjects		
Diet	5.44	**0.025**
Food intake		
Within subjects		
Diet	26.9	<0.001
Glucuronic acid		
Within subjects		
Diet	1.33	0.31
pH		
Within subjects		
Diet	34.0	<0.001
Titratable acid		
Within subjects		
Diet	17.8	<0.001

[a]Degrees of freedom were 2, 10 for all analyses. Significant results are in bold.

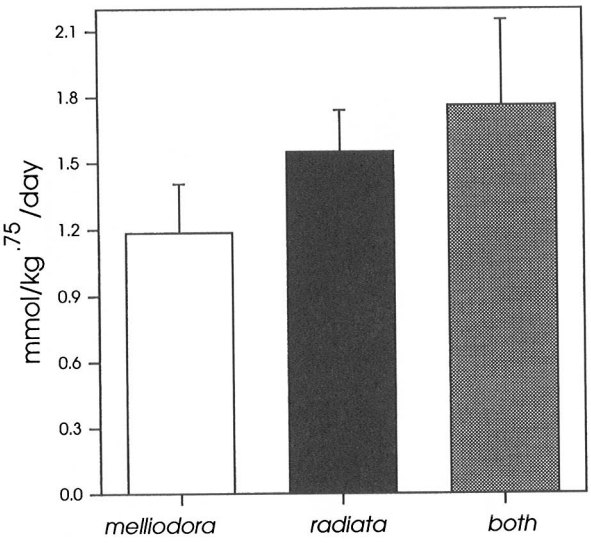

FIG. 3. Mean (+ 1 SE) concentration of glucuronic acid in urine averaged for the last two days of each diet treatment.

The excretion of detoxification metabolites in urine was dependent on both diet treatment and detoxification pathway. There was no difference in the total amount of glucuronic acid excreted on different diets (Figure 3, Table 3). There were differences in urine pH between treatments (Figure 4, Table 3). Animals consuming the *E. radiata* diet produced urine with the lowest pH, possums given both diets produced urine of intermediate pH, and those consuming the *E. melliodora* produced urine with the highest pH (Figure 4). The concentration of titratable acids was significantly greater in the urine of the possums on the *E. radiata* treatment. There was no significant difference in titratable acid levels in animals on the *E. melliodora* and both treatments (Figure 4, Table 3).

Within the diet treatments of *E. radiata* and *E. melliodora* offered singly, daily glucuronic acid production and urine pH were not correlated with daily food intake (Table 4). However, the number of days on each treatment and urine pH were negatively correlated (Table 4). This correlation was most pronounced for animals consuming the *E. radiata* diet. In contrast, glucuronic acid concentrations were not correlated with number of days on the treatment.

DISCUSSION

Freeland and Janzen (1974) hypothesized that mammalian herbivores are forced to be generalist foragers due to their inability to detoxify large quan-

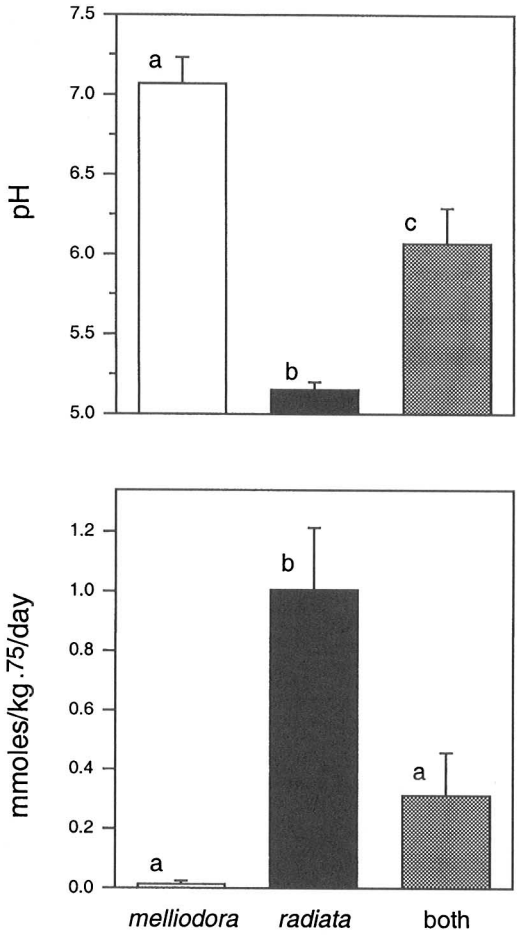

FIG. 4. pH and titratable acids in the urine. The top panel shows urine pH averaged over the last two days of each treatment. The lower panel shows the amount of titratable acids in the urine averaged for the last two days of each treatment. Within a panel, bars with different letters are significantly different. Error bars are one standard error.

tities of similar toxins. Our results provide partial support for this hypothesis. In accordance with the hypothesis, possums ate more food, approximately 33% more, when offered different diets simultaneously versus singly. Production of detoxification metabolites varied as predicted in one (titratable acids) of the two indicators of detoxification. Possums excreted urine with the greatest amounts of titratable acids when consuming *E. radiata* diet, intermediate amounts when feeding on both diets, and the lowest amounts when feeding on *E. melliodora*.

TABLE 4. CORRELATION MATRIX OF FOOD INTAKE, NUMBER OF DAYS A TREATMENT, AND INDICATORS OF DETOXIFICATION FOR POSSUMS CONSUMING *E. melliodora* OR *E. radiata* DIET[a]

	Food intake	Days (*N*)
E. melliodora		
GA	−0.14	0.29
pH	0.35	−0.67*
Food Intake		−0.621*
E. radiata		
GA	0.40	0.369
pH	−0.178	−0.901*
Food Intake		0.11

[a]Significant correlations are marked with asterisks. GA = glucuronic acid production. Because of the multiple comparisons, correlations were not considered significant unless $P < 0.006$.

There were no differences in the excretion of glucuronic acid among possums on the different treatments.

Freeland and Janzen (1974) predicted that when an herbivore consumed the maximum quantity of a particular toxin, individual detoxification pathways became saturated, resulting in negative feedback on food intake. More recently it has been suggested that the elimination routes of detoxification products become saturated and result in a similar negative feedback (Foley, 1992). In particular, excretion of high concentrations of acidic detoxification metabolites in the urine, comparable to those found in possums consuming the *E. radiata* diet in this study, conceivably place an upper limit on the amount of the acid-generating food that can be ingested (Foley, 1992). Possums eliminate terpenes, such as those in *E. radiata*, typically through oxidative processes (Southwell et al., 1980). This reaction yields products that are strong organic acids (Foley, 1992; Foley et al., 1995; McLean et al., 1993). The mammalian kidney has a finite ability for concentrating urine; maximal concentration levels are approximately three orders of magnitude from blood concentrations (Foley et al., 1995). In units of pH, the kidney should approach its maximal concentrating ability at approximately 4.5 (Foley and McArthur, 1994; Foley et al., 1995). After five days on the *E. radiata* diet, possums produced urine with a pH that approached this lower physiological limit. In addition to the difficulties of excreting an extremely acidic urine, metabolic acidosis presents other severe physiological consequences, such as mineral loss from the skeletal system and increased nitrogen loss (Foley and McArthur, 1994; Foley et al., 1995).

The production of acidic detoxification metabolites may have cumulative effects in the long term. During the five-day course of the *E. radiata* treatment, the pH of the urine decreased progressively and dramatically while food intake remained constant. These results suggest a declining ability of possums

to buffer organic acids produced during detoxification. Thus, as buffering stores were depleted, urine pH dropped. Since by the end of day 5 urine pH approached the minimum pH possible, longer term exposure to the *E. radiata* diet may have resulted in a reduction in food intake.

Contrary to our prediction, levels of glucuronic acid in the urine did not differ significantly between possums on the three treatments. Our initial decision to use *E. radiata* and *E. melliodora* was based on previously reported levels of phenolics and terpenes (Foley, 1992; Foley and Hume, 1987). *E. radiata* was reported to have higher terpene concentrations than *E. melliodora*, as we confirmed, and approximately half the concentration of phenolics found in *E. melliodora* (Foley and Hume, 1987). Thus, we had expected that the *E. melliodora* treatment would have phenolic levels double those of the *E. radiata* treatment. Unfortunately, the leaves of *E. radiata* that we used had unusually high concentrations of total phenolics, significantly more than the *E. melliodora* leaves and more than twice the amount reported in the literature (Foley, 1992; Foley and Hume, 1987). In addition, both *E. melliodora* and *E. radiata* had similar high levels of condensed tannins, a subclass of phenolics. Based on intake and phenolic levels of each diet, there were no differences in doses (milligrams of phenolics consumed per day) of either total phenolics or condensed tannins on either diet treatment alone. Therefore, we would not expect to find a difference in glucuronic acid excretion between the two treatments alone. Possums ingested greater doses of total phenolics (15% more) and condensed tannins (22% more) when consuming both diets simultaneously compared with diets fed singly. Although there was no significant statistical difference in glucuronic acid excretion, on both diets, glucuronic acid excretion was 48% higher in possums consuming both treatments compared with *E. melliodora* alone and 14% higher on both diets compared with *E. radiata* alone (Figure 3). The large variation in glucuronic acid excretion between individuals may have hindered our ability to detect differences.

Metabolites of both the phase 1 and phase 2 pathways contribute to the total titratable acid in the urine. Although we did not identify individual detoxification metabolites, we credit the high concentration of titratable acid in the urine of possums on the *E. radiata* treatment to the phase 1 pathway based on the following evidence. The predominant phase 2 pathway in marsupials is the glucuronic acid pathway (Baudinette et al., 1980). Levels of glucuronic acid did not differ tremendously among possums on the various diet treatments and thus cannot account for the large increases in levels of titratable acid in animals on the *E. radiata* treatment. Moreover, investigations of marsupials consuming either individual terpenes found in *E. radiata* or entire leaves of *E. radiata* have demonstrated that terpenes are detoxified through the phase 1 pathway (McLean et al., 1993; Southwell et al., 1980).

Is there evidence that either detoxification route (phase 1 or phase 2 glucuronidation) measured in this experiment became saturated? Our data suggest that

when possums were consuming the *E. radiata* diet alone, phase 1 detoxification may have been limited by the generation and elimination of acidic detoxification metabolites. On this diet, urine pH approached the minimum pH that mammals can excrete (Foley and McArthur, 1994; Foley et al., 1995). Detoxification limitations of possums on the *E. melliodora* and both diet treatments are more difficult to attribute to either pathway measured here. Assuming that there was no change in glucuronic acid production on the three treatments, as suggested by the statistical analysis, it is plausible that the glucuronic acid pathway was saturated on all three diets. The physiological limits of glucuronic acid production by possums is unknown. Therefore, it is impossible to say whether the amount of glucuronic acid produced was at saturation levels. Lastly, mammals possess conjugation pathways other than the glucuronic acid pathway. We chose to focus on the glucuronidation pathway because it is the predominant one in marsupials (Baudinette et al., 1980). We cannot exclude the possibility that a phase 2 pathway other than the glucuronic acid was saturated and responsible for limiting food intake.

The quantity of *Eucalyptus* leaves in the treatment diets prevented possums from maintaining body mass. Thus, they represent potential maximal amounts of each eucalypt species that possums can tolerate. In nature, when food items other than *Eucalyptus* are available, the amount of *Eucalyptus* consumed may be considerably less than that consumed in this study. Wild possums tend to consume a variety of food items each night (Freeland and Winter, 1975). The incorporation of many food items in the diet, particularly those lower in secondary compounds than *Eucalyptus*, e.g., grasses, forbs, fruits (Fitzgerald, 1978; Kerle, 1984), may substantially reduce impacts, especially long-term ones, to the detoxification system. Such a foraging strategy may act to maximize the net nutritional content extracted from the diet while simultaneously minimizing costs of detoxification.

To test the detoxification-limitations hypothesis without the potentially confounding effects of differences in nutrient content, we attempted to hold nutrient levels constant among treatments by using species of eucalypts with similar nutritional contents. Fiber did not differ between the two species. Nitrogen differed slightly but significantly between leaves of the two species. However, the difference in nitrogen content does not invalidate our conclusions. *Eucalyptus* leaf comprised 50% of each diet treatment (Table 1), thus, the difference in nitrogen content between the *E. radiata* and *E. melliodora* treatments was 0.06%. We do not know whether possums can discriminate between diets differing at these nitrogen levels. We found no evidence in our experiments that they did. Although the *E. radiata* treatment was slightly higher in nitrogen, possums consumed equal amounts of the *E. radiata* or *E. melliodora* treatments when offered singly. Moreover, on all treatments, food intake was below maintenance intake, as evidenced by weight loss. This result suggests that food intake was not being regulated by nitrogen intake but rather was being limited by inhibiting agents, such as plant secondary compounds.

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IDENTIFICATION OF A NEW BLEND OF APPLE VOLATILES ATTRACTIVE TO THE APPLE MAGGOT, *Rhagoletis pomonella*

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Abstract—Solid-phase microextraction (SPME) and gas chromatography coupled with electroantennographic detection (GC-EAD) were used to identify a new blend of volatiles from apples as the key attractants for the apple maggot fly, *Rhagoletis pomonella* (Walsh). The new five-component blend contains butyl butanoate (10%), propyl hexanoate (4%), butyl hexanoate (37%), hexyl butanoate (44%), and pentyl hexanoate (5%) compared with a previously reported seven-component mix of hexyl acetate (35%), (*E*)-2-hexen-1-yl acetate (2%), butyl 2-methylbutanoate (8%), propyl hexanoate (12%), hexyl propanoate (5%), butyl hexanoate (28%), and hexyl butanoate (10%). Volatiles from five different varieties of apple elicited reproducible and high EAD responses from *R. pomonella* antennae to the same five chemicals. In flight-tunnel choice tests involving red sticky spheres with odor sources, the new five-component blend of apple volatiles showed significantly more activity than the previous seven-component blend or the single compound, butyl hexanoate. In a field trial captures with the new five-component blend were better than with butyl hexanoate, which is currently used with commercial apple maggot monitoring spheres.

Key Words—*Rhagoletis pomonella*, apple maggot fruit fly, solid-phase microextraction, gas chromatographic–electroantennographic detection, flight tunnel, host volatiles, field experiments.

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INTRODUCTION

In fruit flies belonging to the *Rhagoletis pomonella* species complex, it has been suggested that host odors are critical for host location and fidelity and that shifts in host odor specificity may be involved in sympatric speciation within the group (Feder and Bush, 1989; Frey and Bush, 1990; Bush, 1993, 1994; Feder et al., 1994). One of the most studied species in the complex is the apple maggot, *R. pomonella* (Walsh), which occurs in North America on native hawthorne (*Crataegus*), the natural host, and on introduced apple (Chapman and Lienk, 1971; Fletcher and Prokopy, 1991). Prokopy et al. (1973) showed that in a field situation *R. pomonella* flies were attracted to the odor of apples, which led to the characterization of a seven-component mix (Fein et al., 1982) containing hexyl acetate, (*E*)-2-hexen-1-yl acetate, butyl 2-methylbutanoate, propyl hexanoate, hexyl propanoate, butyl hexanoate, and hexyl butanoate from apple extracts (see Carle et al., 1987). This blend elicited positive behavioral responses in olfactometer tubes and a wind tunnel (Fein et al., 1982; Averill et al., 1988). Field tests showed also that the chemical mix was an effective attractant (Reissig et al., 1982).

Here, we present evidence that a new five-component blend of volatiles from apples proved to be more attractive than the previous blend to *R. pomonella* flies in the laboratory and in the field. The new blend was identified by using a combination of two techniques in the analysis of headspace volatiles from fruit: solid-phase microextraction (SPME; Supelco) (see Bartelt, 1997) combined with gas chromatography and electroantennographic detection (GC-EAD).

METHODS AND MATERIALS

Insects. Adult *R. pomonella* were obtained from a laboratory colony maintained on Red Delicious apples (Neilson and McAllen, 1965). Pupae were kept in an environmental chamber with 24°C day and 22°C night temperatures, 16L : 8D photoperiod, and 35% relative humidity. Within one week after fly emergence, the adults were transferred to a second chamber with 23°C constant temperature, 50% relative humidity, and 16L : 8D photoperiod. Adults were maintained on an artificial diet (water, sugar, vitamins, casein hydrolysate, and salt mixture), and larvae were reared on Red Delicious apples. Adult flies at 0–7 days old and 2–3 weeks old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively.

Fruits. Five different apple varieties (Empire, Crispin, Cortland, Macintosh, and Red Delicious) were collected from uninfested orchards near Geneva, New York, in the 1996 harvest season and stored at 4°C. The fruits were taken from cold storage and placed in the sampling containers (see below) at room

temperature at least 1 hr before the SMPE sampling began. At least four samples were collected from each variety.

SPME Sampling. Single apples were placed in 500-ml clear straight-sided jars with Teflon liner screwcaps (Wheaton, Millville, New Jersey). A polydimethyl siloxane-coated SPME fiber (film thickness 100 μm; Supelco Inc., Bellefonte, Pennsylvania) was conditioned in the GC injector (250°C) for 5 min and then passed through the small hole on the cap into the jar. The fiber was exposed for 10 min to absorb the volatiles, and the needle was then pierced into the GC injection port for 2 min to desorb the analytes.

Electrophysiological Recordings. Coupled GC-EAD analyses were performed as described before (Zhang et al., 1997). A Hewlett Packard 5890 Series II gas chromatograph equipped with a nonpolar SE-30 Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Alltech Associates, Inc., Deerfield, Illinois) or a polar Stabilwax capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Restek Corp., Bellefonte, Pennsylvania) in the splitless mode was used for GC-EAD analysis. The oven temperature was programmed at 50°C for 2 min, then 10°C/min until 250°C, and held for 20 min. Injector and detector temperature were set at 250°C. Nitrogen was the carrier gas with a flow rate of 2 ml/min. Septum purge flow rate was set at 3 ml/min and the split vent flow rate at 2 ml/min. All of the gas source pressures were 276 kPa (40 psi), and initial column head pressures were 138 kPa (20 psi). The column effluent was split 1 : 1 in the oven of the flame ionization detector and the EAD. A whole head was removed from a fly and both antennae were positioned between two gold wire electrodes, which were immersed in saline-filled wells in a small acrylic holding station. The fly's head was placed in one well and the tips of both antennae maneuvered to make contact with the saline in the other well. The temperature of the antennae on the acrylic station was maintained at about 5°C by flushing 0°C water from a benchtop refrigerated circulator (RTE-100, NESLAB Instruments, Inc., Portsmouth, New Hampshire) through the insulation layer of the modified condenser containing the acrylic station mounted on top of the GC. An HP 3390 A integrator was used for the EAD recordings. A minimum of 10 different antennal pairs (2–3 runs/pair) were used to analyze volatiles from each cultivar.

GC-mass spectrometry (GC-MS) was carried out with a Hewlett Packard 5890 gas chromatograph coupled to an HP 5970B Mass Selective Detector (EI) with an SE-30 capillary column, but with helium as the carrier gas. The oven temperature was programmed at 40°C for 5 min, then 5°C/min to 250°C, and held for 20 min.

Chemicals. All synthetic compounds were purchased from either Pfaltz & Bauer, Inc., Stamford, Connecticut, or Penta International Corp., West Caldwell, New Jersey, and purities were >98% based on results with the capillary GC (FID).

TABLE 1. APPLE MAGGOT ATTRACTANT BLENDS USED IN FLIGHT TUNNEL (FT) AND FIELD TESTS

Old blend		New		
Compound	FT (%)	Compound	FT (%)	Field
Hexyl acetate	35%			
(*E*)-2-Hexen-1-yl acetate	2%			
		Butyl butanoate	10%	2
Butyl 2-methylbutanoate	8%			
Propyl hexanoate	12%	Propyl hexanoate	4%	1
Hexyl propanoate	5%			
Butyl hexanoate	28%	Butyl hexanoate	37%	10
Hexyl butanoate	10%	Hexyl butanoate	44%	10
		Pentyl hexanoate	5%	1

Flight Tunnel. Responses of AM flies to host volatiles were measured in a sustained-flight tunnel (Glover et al., 1987). Outside dimensions of the tunnel were 183 cm long, 61 × 61 cm square; the tunnel was constructed of glass and had a metal frame. Diffuse overhead light was provided by four Vita-Lite fluorescent bulbs (Fein et al., 1982), as well as fluorescent lighting from the ceiling of the room housing the tunnel. The tunnel was housed in a room with temperature and relative humidity controls and an exhaust system from the tunnel to the outside of the building. Flight-tunnel conditions were: 25–27°C, 50–70% relative humidity, 40 cm/sec wind speed, and 1200 lux light intensity.

The experimental design involved a choice test, modified after Fein et al. (1982) and Averill et al. (1987), incorporating red spheres (7.5 cm; Gempler's Inc., Mt. Horeb, Wisconsin) coated with Tangle trap. One sphere served as a blank or as a second odor source. The two spheres were hung from the roof of the upwind end of the tunnel by metal wires so that they were 25 cm above the tunnel floor and 35 cm apart. Treatments included the seven-component blend, the new five-component blend, and the single compound butyl hexanoate. The single compound or the mixtures (see Table 1) were prepared in hexane at concentrations so that 100 μl of solution were applied for each load rate to red rubber septa (Thomas Scientific, Swedesboro, New Jersey, Cat. No. 1780J07) that had been acetone-washed. Sources were prepared 30 min prior to a test and were clipped onto the metal rods holding the spheres, 1 cm above a sphere. Blank spheres contained rubber septa treated with hexane.

Two flight tunnel experiments were conducted. The first involved choice tests between a blank sphere and one with 10, 30, 100, or 300 μg of the seven-component blend, the new five-component blend, or the single compound butyl hexanoate. The second involved choice tests between spheres containing 100-μg doses of the five-component blend and the seven-component blend or butyl hex-

anoate alone. For each test, 50 flies (mixed sex, 2–3 weeks old) were placed in glass vials (10–15 flies/vial). The flies were selected from holding cages located in a separate, environmentally controlled, room, taken to the flight tunnel, and allowed to acclimate for 5 min. The odor source(s) were then placed in the tunnel and the flies released. Fresh volatile sources and red spheres were used for each test. Tests ran for 1 hr, at which time the number of captured flies was recorded. For all tests, six replicates were performed, with the position of the spheres alternated over the six replicates.

Field Experiments. A field test involving the new apple blend and butyl hexanoate, the commercial treatment, was conducted during July, August, and September, 1997, in Massachusetts. The test involved eight commercial apple orchards, divided into two groups of four orchards each, group 1 trees were baited with butyl hexanoate for the first half of the test (four weeks), and then with the new apple blend (four weeks), whereas group 2 trees were baited with butyl hexanoate throughout the season (eight weeks). The new apple blend, a 2:2:10:10:1 mixture of butyl butanoate, propyl hexanoate, hexyl butanoate, butyl hexanoate, and pentyl hexanoate, was loaded to capacity in 2-dram polyethylene vials (Reissig et al., 1982), similar to the butyl hexanoate lures. The proportions approximated the average values obtained from integration of the total ion mass spectra obtained with a temperature program that gave separation of all compounds from the five apple varieties (see Table 2 below). For each orchard, there were three blocks of 49 trees in a 7×7 arrangement. Each 49-tree block was at the corner of a larger block, the remainder of which was sprayed three times with insecticide for apple maggot fly control during the summer of 1997. Thus, for each 49-tree block, two sides faced adjacent woods and two sides faced sprayed trees. Each of the 26 perimeter trees in each 49-tree block received a baited sticky red sphere. All spheres were in place in early July. Counts were made twice during each half of the test.

Statistical Analysis. Mean capture rates for the six replicates in the flight tunnel and for the two treatment groups in the field were compared by using a two-sample independent groups t test, with GB-STAT for Macintosh ($P < 0.05$). The analysis included tests for normality and homogeneity of variance, according to the GB-STAT program.

RESULTS

Identification of Key Host Volatiles from Apples. For each apple variety, SPME headspace sampling and GC-EAD analysis was conducted at least four times by using male and female antennae. Although the GC profiles varied among the apple varieties, SPME sampling of five different varieties consistently revealed the same four significant antennal responses to the volatiles on

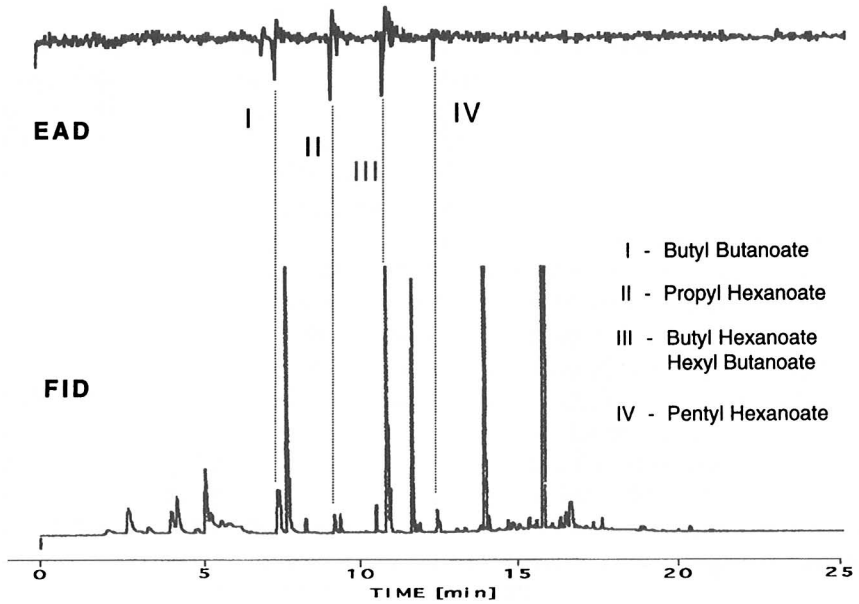

FIG. 1. Simultaneous responses of FID and EAD using the antennae of female *R. pomonella* to SPME desorbed apple volatiles on an SE-30 capillary column. The trace is from an Empire apple, but is representative of the five varieties sampled.

the SE-30 and the Stabilwax capillary columns (Figure 1). No significant difference in antennal sensitivity was observed between male and female antennae. The corresponding EAD active compounds were identified as butyl butanoate (peak 1), propyl hexanoate (peak 2), butyl hexanoate and hexyl butanoate (peak 3), and pentyl hexanoate (peak 4), respectively, by comparison of mass spectra and GC-MS retention times on both polar and nonpolar capillary columns with synthetic standards. Although butyl hexanoate and hexyl butanoate cochromatographed as the third EAD active peak under the GC conditions used, the two compounds could be separated by using a lower temperature program without any loss in EAD response. These two compounds made up about 80% of the total five active esters and usually elicited the strongest GC-EAD response from headspace volatiles. The relative ratios of the five esters in the different apple varieties, as determined by SPME and GC-MS, are listed in Table 2. Activity of the five esters was confirmed by strong antennal responses elicited by 1 ng or less of synthetic compounds.

Flight Tunnel Experiment 1. Upon exposure to the odor stream, flies emerged quickly from the vials and dispersed in the downwind end of the tunnel. Capture on the red sphere occurred via a series of short flights biased in

TABLE 2. RATIO OF ACTIVE CHEMICALS FROM DIFFERENT APPLE VARIETIES AS DETERMINED BY INTEGRATION OF GC-MS TOTAL ION CURRENT[a]

Chemicals	Apples					Mean ± SD
	Em	Cr	Co	Mc	RD	
Butyle butanoate	4.3	9.4	8.4	24	15.9	12.4 ± 7.7
Propyl hexanoate	6.7	3.5	3.5	5.8	6.3	5.2 ± 1.5
Butyl hexanoate	83	75	17	25	25	45 ± 31.3
Hexyl butanoate	17	25	83	75	75	55 ± 31.1
Pentyl hexanoate	9.8	4.8	8.4	3.5	4.5	6.2 ± 2.7

[a]Apples: Em = Empire, Cr = Crispin, Co = Cortland, Mc = Macintosh, RD = Red Delicious. Ratios calculated on the basis of butyl hexanoate and hexyl butanoate, which cochromatograph on the GC-EAD, summing to a value of 100. At least four samples were collected from each variety.

the upwind direction, with less than 5% of the flies exhibiting directed flights at distances of 1.0 m or greater to the sphere. Short flights were interspersed with bouts of walking and grooming. Short flights, grooming, walking, and encounters with other flies appear to be basic components of normal arousal behavior of the flies during the photophase. The presence of odor increased the rate of net upwind movement bringing adults to the upwind end of the tunnel, where in numerous instances short directed flights were made to the spheres. The success of the assay may have been due to the small height and width of the tunnel (61 × 61 cm), which kept the flies within or close to the odor space for the duration of the test.

In the first experiment, adult responses were assessed to one of four doses (10, 30, 100, or 300 μg) of the new or old apple blend, or butyl hexanoate, against a blank sphere. The results of these choice tests (Figure 2), show that mean captures on the blank spheres were consistent over the entire set of tests, averaging 6 ± 2 flies/sphere, and with no significant differences among the blanks for any dose/treatment combination. With the 10-μg sources, there was no significant difference in mean captures among the three treatments and the blanks. Greatest response at 30, 100, and 300 μg was observed with the new apple volatile blend. Mean values with the new blend were significantly greater than controls at 30 μg, and at 100 and 300 μg this was true for all three treatments compared with the blanks.

Flight Tunnel Experiment 2. Adult responses were assessed using the 100-μg dose in choice tests involving either the new five-component vs. the seven-component apple blend or the new blend vs. butyl hexanoate. The 100-μg dose was selected based on differences in mean captures observed in experiment 1. The results (Figure 3) show that in each choice test significantly more flies were captured with the new apple blend.

Field Trial. The results of field tests comparing fly responses to the new

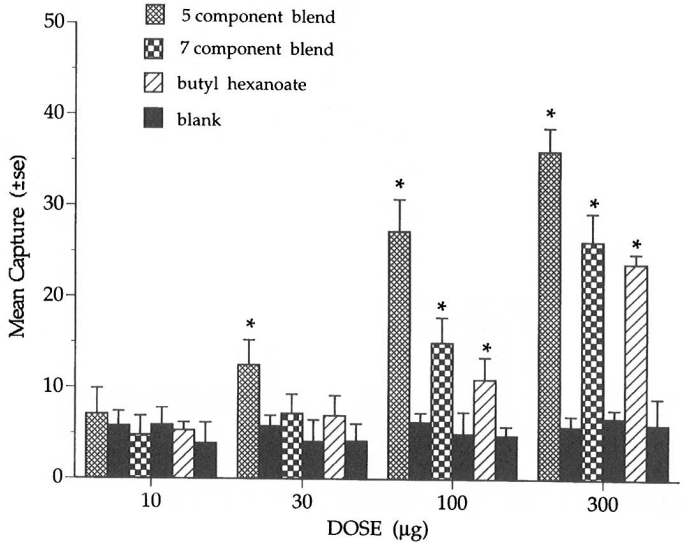

FIG. 2. Responses of adult *R. pomonella* in a sustained-flight tunnel in choice tests involving four doses (10, 30, 100, and 300 μg on rubber septa) of the new five-component blend, the seven-component blend, or butyl hexanoate on a sticky red sphere versus a blank red sphere. Values are mean capture ± SE, $N = 6$ replicates of 50 flies for each choice test. Bars within each choice test with different letters indicate significant differences, by using a t test ($P < 0.05$).

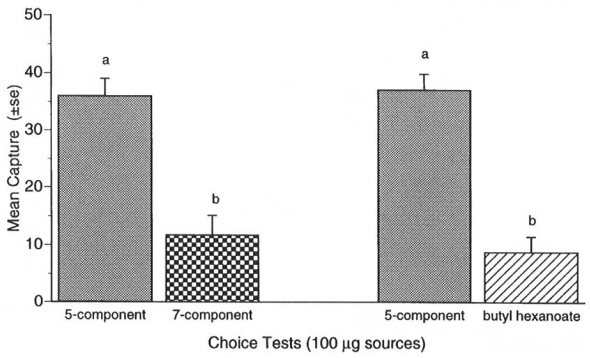

FIG. 3. Responses of adult *R. pomonella* in flight tunnel choice tests to the new five-component blend versus the seven-component blend (left), and the new blend versus butyl hexanoate (right). Values are mean ± SE, $N = 6$ replicates of 50 flies for each test. Bars within each choice test with different letters indicate significant differences, by using a t test ($P < 0.05$).

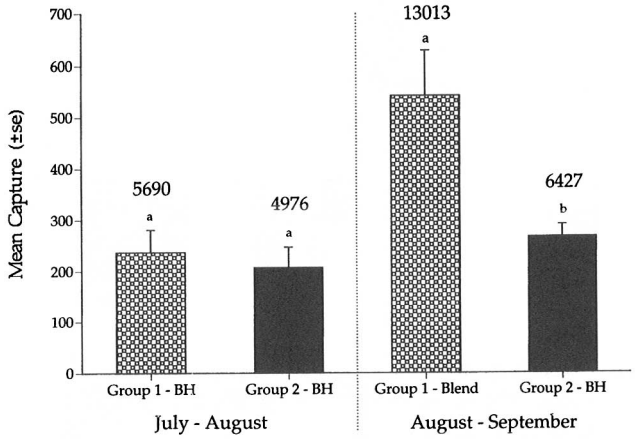

FIG. 4. Mean capture (± SE) of adult *R. pomonella* in field trials comparing sticky red spheres baited with two butyl hexanoate treatments (groups 1 and 2 in July–August on the left), and the new five-component blend (group 1) versus butyl hexanoate (group 2) in August–September (on the right). Numbers above each bar are the total number of flies captured. Bars in each test with different letters indicate significant differences, by using a t test ($P < 0.05$).

apple blend and butyl hexanoate are shown in Figure 4. During July and early August, tests were performed in which red spheres in both groups of orchards were baited with butyl hexanoate. There was no significant difference in mean captures between the two groups. In late August–September, red spheres in orchards in group 1 were baited with the new apple blend, and mean capture in this group was significantly greater than in the orchards in group 2 baited with butyl hexanoate.

DISCUSSION

We have identified a new five-component blend of volatiles from apples that contains three compounds (propyl hexanoate, butyl hexanoate, and hexyl butanoate) in common with the previous seven-component blend, as well as two new compounds, butyl butanoate and pentyl butanoate. The esters in the new blend were identified from five apple varieties and gave consistently high levels of EAD activity. Flight-tunnel experiments showed that the new blend was more attractive to *R. pomonella* adults than the previously identified blend of esters or the single compound, butyl hexanoate; field experiments showed that the new mix was superior to butyl hexanoate.

Successful characterization of apple volatiles resulted from the use of SPME

and GC-EAD. The SPME technique deletes the solvent extraction step, allowing for collection of volatiles directly from the headspace of the fruit in quantities that are consistent with their release rate in the field and without interference from contaminating compounds typically found in collections that use more traditional methods (see Bartelt, 1997; Agelopoulos and Pickett, 1998; Matich et al., 1996, for recent critical discussions of the SPME technique). Often, high quantities of fruit volatiles are collected by other methods, with the GC-EAD analysis exhibiting mediocre responses to many compounds and no clear evidence for the key components. With our experimental conditions, the amount of each electrophysiologically active compound collected by SPME was in the range of 1–20 ng based on GC analysis.

One potential criticism of our analysis concerns the use of apples that were picked and kept in cold storage, or, in general, the problem of changing volatile profiles as a function of age or picking. In a previous study, Carle et al. (1987) analyzed volatile profiles from a number of apple varieties and showed that, whereas many of the same esters occurred in each variety, there was significant variability in the profiles for each variety and that these profiles changed quantitatively over the growing season and with respect to storage conditions. Sampling the volatiles, however, with the SPME technique and analysis of these volatiles with GC-EAD showed that the same key compounds are found, regardless of the qualitative and quantitative differences in the volatile profiles.

The sustained-flight tunnel was used to assay the identified compounds for behavioral activity, with a 1-hr period proving sufficient to test the ability of *R. pomonella* flies to respond to and discriminate between host odors. The sustained-flight tunnel had been used previously with *R. pomonella* by Fein et al. (1982) for the characterization of the seven-component apple blend and by Aluja et al. (1993) to study the effects of air speed and volatiles on *R. pomonella* behavior. Although our flight tunnel did not incorporate structures simulating foliage (as in Aluja et al., 1993), the flies exhibited patterns of behavior similar to those that have been observed in field cage experiments demonstrating the effect of host volatiles on inter- and intratree flight behaviors (Aluja and Prokopy, 1992, 1993; Aluja et al., 1993; Green et al., 1994).

The new five-component blend of esters also was attractive in apple orchards, capturing more *R. pomonella* on red sticky spheres than equivalent amounts of butyl hexanoate alone. The latter compound is often used in conjunction with red spheres as a monitoring tool in commercial orchards.

A major goal of our research is to address questions about the role of host odors in maintaining host specificity in *Rhagoletis* flies and in the proposed processes involving host shifts via sympatric speciation. The dependence of the mate recognition system on the linkage between host choice and mate finding makes it possible for variation in traits affecting host utilization to act as genetically based, premating isolation mechanisms (McPheron et al., 1988; Feder et al.,

1989, 1990; Bush, 1993, 1994; Berlocher et al., 1993; Smith and Bush, 1997). With the development of the SPME and GC-EAD techniques, it should be possible to identify the key host volatiles for species within the *R. pomonella* group and to test in greater detail questions concerning the role of host odors.

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AN OVIPOSITION STIMULANT FOR SPICEBUSH SWALLOWTAIL BUTTERFLY, *Papilio troilus*, FROM LEAVES OF *Sassafras albidum*

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Abstract—Female butterflies of the spicebush swallowtail, *Papilio troilus*, are specialists, ovipositing on plants in the family Lauraceae. Column chromatography and HPLC were used to isolate an oviposition stimulant from the leaves of one of its hosts, *Sassafras albidum*. The stimulant was identified as 3-*trans*-caffeoyl-muco-quinic acid on the basis of FAB-MS and ^{1}H NMR spectra as compared to a compound previously isolated from another plant. It was not active alone, but it increased the oviposition activity of butterflies when combined with other stimulant(s) at a concentration of 7 ng/mm^2 leaf surface area. Other caffeoylquinic acid isomers tested did not have this effect. This is the first report of a swallowtail contact oviposition stimulant from a plant in the family Lauraceae.

Key Words—Swallowtail butterfly, Papilionidae, *Papilio troilus*, Lepidoptera, Lauraceae, *Sassafras albidum*, oviposition stimulant, 3-*trans*-caffeoyl-muco-quinic acid.

INTRODUCTION

The spicebush swallowtail, *Papilio troilus* L. (Lepidoptera: Papilionidae), is one of three species in the *P. troilus* species group (Munroe, 1961; Hagen and Scriber, 1991; Scriber et al., 1991; Sperling, 1993). Female butterflies in this species

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group oviposit only on plants in the family Lauraceae (Scriber, 1984; Scriber et al., 1991). The range of the spicebush swallowtail is widespread, from central Florida north to Massachusetts and west through lower Michigan, Illinois, and eastern Oklahoma and Texas, with occasional records farther west (Klots, 1951; Howe, 1975; Scott, 1986). Its typical habitat is temperate deciduous forest (Tyler and Brown, 1994). Two subspecies have been described (Hagen and Scriber, 1991). The range of the nominate subspecies *P. t. troilus*, which we have studied here, overlaps the coexisting range of one of its major host plants, common sassafras [*Sassafras albidum* (Nutt.) Nees] (Nitao et al., 1991).

Extensive studies of the oviposition chemistry of three other *Papilio* species, *P. xuthus*, *P. protenor*, and *P. polyxenes*, have found the key classes of contact oviposition stimulants from plants in the families Rutaceae and Apiaceae (Umbelliferae) to be flavonoid glycosides (Feeny et al., 1988; Honda, 1986; Nishida, 1989; Ohsugi et al., 1991), hydroxycinnamic acids (Feeny et al., 1983, 1988; Honda, 1990), and basic and neutral compounds (Carter et al., 1998; Nishida et al., 1987, 1990; Nishida and Fukami, 1989; Oshugi, 1991). This study was undertaken to determine if similar or different classes of compounds would be used by a swallowtail in the genus *Papilio* that oviposits on plants in a more distantly-related plant family, the Lauraceae.

METHODS AND MATERIALS

Insects. There were two sources of adult butterflies for bioassay experiments. From January to May, adults emerged from diapausing pupae purchased from amateur suppliers in the northeastern United States. Diapausing pupae were removed from cold treatment (4°C) and placed in a large walk-in growth chamber with day/night temperatures of 29/16°C and a photoperiod of 16L : 8D. Eclosing adults were allowed to dry for 24 hr before hand-feeding and hand-pairing (Carter and Feeny, 1985). Butterflies were fed daily with a solution of approximately 1 part honey in 10 parts modified Ringer's solution (Lederhouse et al., 1990). Mated female and male butterflies were allowed to fly freely in the growth chamber and nectar on colored sponges in Petri dishes filled with fresh feeding solution and on a variety of potted flowering plants. The walls of the growth chamber were covered with netting to provide a resting surface.

During the summer months spicebush swallowtail caterpillars originating as eggs from the above adults were reared on sassafras foliage either in net bags in the field or on foliage in water-filled aquapics placed in plastic boxes in the growth chamber.

Bioassay Design. Female butterflies were allowed to oviposit on fresh sassafras foliage presented either as small potted trees or as a cut branch placed in the center of a table (1.5 × 2 m) in the middle of the growth chamber. On a

test day, foliage was covered with a net cage in the late morning. Trials were conducted in the early afternoon and lasted 2–3 hr. Six to 20 mated female butterflies were present in the growth chamber during each trial. The oviposition substrate was a fava bean plant (Broad Windsor, 10–14 days old). Volumes of test fractions, solutions of pure compounds, or solvents were transferred with an HPLC syringe and placed into a small (5 ml) TLC reagent sprayer. The test solutions were then sprayed onto the upper surfaces of the leaves of the bean plant and allowed to dry.

For each trial within an experiment, two experimental plants, treated with test solutions, and two control plants, treated with the solvents MeOH and H_2O, were placed alternately on the four corners of the table. Plants were rotated around the table in a clockwise direction every 30 min. At the end of a trial, eggs laid on the bean plants were counted. The net cage was removed from the sassafras foliage to allow females to oviposit until the following day. For statistical comparisons, an experiment comprised results from six trials. Egg numbers from the two control plants were averaged to compare with egg numbers laid on each of the treatment plants in a trial.

Data were analyzed by a paired t test for the mean proportion of eggs laid on either the treatment plants or the control plants as compared to the expected value of 0.5, assuming that no oviposition preference exists between treatment and control. If two different treatments were present during an experiment, the means were compared directly by a paired t test.

Initial Extraction and Fractionation of Plant Material. Sassafras foliage was collected in Tompkins County, New York. The leaves were extracted with boiling 95% EtOH in a blender and boiled for 5 min before being filtered. The extract was reduced by rotary evaporation at <50°C to remove EtOH and centrifuged to remove chlorophyll. The resulting aqueous (aq.) layer was extracted sequentially with three volumes each of the following organic solvents: hexane, Et_2O, $CHCl_3$, and EtOAc. The solvent extracts were reduced by rotary evaporation to dryness, and the remaining material was dissolved in MeOH at 5 gle (gram leaf equivalents, wet weight)/ml. The residual post-EtOAc aqueous layer was reduced to 5 gle/ml in H_2O.

Column Chromatography of Aqueous Post-EtOAc Extract. Twenty-five gram leaf equivalents of the post-EtOAc aq. extract were separated on a flash column (19 × 150 mm; C18 Bonded Phase, 40 μm). The column was eluted with 300 ml of 1% aq. AcOH (fraction A); 300 ml of 20% MeOH in 1% aq. AcOH (fractions B1–B6); 400 ml of 40% MeOH in 1% aq. AcOH (fraction C), and 200 ml of 100% MeOH (fraction D). Fractions were reduced by rotary evaporation and brought to final concentrations of 5 gle/ml with H_2O (fraction A) or 50% MeOH (aq.) (B, C, and D). For preparative isolation of the active stimulants, 200 gle of the post-EtOAc aq. layer was chromatographed similarly on a larger column. B fractions (Prep. B1–Prep. B5) were collected in 1000 ml

of 20% MeOH in 1% aq. AcOH. These were analyzed on an IBSIL RP C-18 HPLC column (5 μm; 250 × 10 mm) by using the following solvent gradient (system 1; flow rate 3 ml/min; curve 6) monitored by UV at λ_{254}: 100% of 1% aq. AcOH to 80% of 1% aq. AcOH/20% MeOH for 15 min; to 65% of 1% aq. AcOH/35% MeOH for 25 min; to 100% MeOH for 10 min.

Analytical Column Chromatography of Active B Subfractions. The B fractions found to be active in bioassay were further chromatographed on a flash column packed with CHP20P (14 × 150 mm; 75–150 μm) and eluted with a gradient of 10% MeOH in 1% aq. AcOH to 45% MeOH in 1% aq. AcOH, increasing the proportion of MeOH by 5% increments. Fractions were collected in volumes of 2.5 ml and reduced further to 1 ml under a stream of nitrogen. All fractions were spotted on HPTLC plates [5 × 5 cm, silica gel with UV (λ_{254}) indicator] developed in butanol–acetic acid–water (BAW, 4 : 1 : 5; top layer) and visualized under UV (λ_{254} and λ_{365}) light. Fractions were also monitored by HPLC (system 1).

Purification of Active Caffeoylquinic Acid. The precipitate in one of the active B fractions was cleansed by repeated washing and centrifuging with cold water. This precipitate, labeled compound X, was dried under nitrogen. When needed, compound X was weighed and dissolved in MeOH–water (50 : 50). To check for purity, a solution of compound X was chromatographed on HPLC (system 1) and the amount needed in bioassay was calculated from the area count of the same peak in active CHP column fractions.

Isolation of Caffeoylquinic Acid Isomers Prepared from Chlorogenic Acid for Bioassay Experiments. A mixture of caffeoylquinic acid isomers was obtained from 5-caffeoylquinic acid (chlorogenic acid) (Nagels et al., 1980). The mixture of isomers was chromatographed on a flash column (19 × 220 cm) packed with C18 (Bonded Phase; 40 μm), and eluted with a gradient from 1% aq. AcOH to 30% MeOH in 1% aq. AcOH in increments of 5% MeOH. Separation and purity of the caffeoylquinic acid isomers were monitored by HPLC (system 1). One hundred microliters of a standard solution of 5-caffeoylquinic acid was prepared in 50% aq. MeOH for bioassay experiments and checked for purity by HPLC (system 1).

Instrumentation and Methods for Identification of Active Caffeoylquinic Acid Derivative. Low-resolution FAB-MS of compound X was recorded on a VG ZAB-SE reverse geometry ultrahigh resolution mass spectrometer equipped with a FAB ion source and a VG OPUS data processing system. ^{1}H NMR spectra of the active stimulant and the standard were recorded on a 500-MHz instrument in DMSO-d_6.

Calculation of Bean Plant Leaf Surface Area. Individual bean plants used to test the pure oviposition stimulant were weighed by cutting each stem off at the soil line and placing it on a balance. The wet weights of a second group of 50 bean plants were recorded similarly, and for these plants the leaves were cut

from the stems and the upper surface area of each leaf was recorded from a LI-COR LI-3100 area meter. Surface area measurements of leaves from the same bean plant were added. A regression equation (model I) was calculated from the plant wet weight and leaf surface area recordings to estimate surface area as a function of plant wet weight for the leaves of the bean plants treated with the isolated oviposition stimulant.

RESULTS

Activity of Sassafras Ethanolic Extract and Fractions. Bean plants sprayed with an alcoholic extract of sassafras leaves received significantly more eggs than did bean plants sprayed with solvent alone (Table 1, experiment 1). After fractionation of the ethanolic extract, the post-EtOAc aq. extract alone was found to be significantly more active than the control, and oviposition activity was not enhanced by the addition of the recombined organic extracts (Table 1, experiment 2).

Activity of Fractions of Post-EtOAc Aqueous Extract. The C18 column chromatography fractions were bioassayed in a logical progression (Table 2). Fraction A was not active alone (experiment 1). Only three eggs were laid in all six trials. B1–B6 were combined for bioassay as fraction B. This was found to be highly active alone, and its activity was not enhanced significantly by addition of A (Table 2, experiment 2). Fractions C+D did not increase the activity of A+B (Table 2, experiment 3). We concluded, therefore, that most of the activity of the parent ethanolic fraction was present in fraction B.

TABLE 1. OVIPOSITION RESPONSES OF FEMALE SPICEBUSH SWALLOWTAIL BUTTERFLIES TO ALCOHOLIC EXTRACT OF SASSAFRAS LEAVES AND ITS AQUEOUS AND NONPOLAR (COMBINED ORGANIC SOLVENT) FRACTIONS[a]

Treatment 1	$\bar{X}$[b]	Eggs[c]	Treatment 2	$\bar{X}$	Eggs	P[d]
Exp. 1. Sassafras alcoholic extract (1.0 gle)						
Control	0.11	40	Alc. Ext.	0.89	326	0.001
Exp. 2. Post-EtOAc aqueous extract (2.5 gle) vs recombination of post-EtOAc and organic fractions (2.5 gle each)						
Control	0.07	37	P.-EtOAc	0.93	500	0.001
Control	0.08	37	P.-EtOAc + Org.	0.92	451	0.001
P.-EtOAc	0.51	500	P.-EtOAc + Org.	0.49	451	NS

[a]Each experiment combined the results of six replicated trials (N = 6–20 females/trial; NS = not significant, $P \geq 0.05$).
[b]Mean ($\bar{X}$) proportion of eggs laid on each of the two treatments in a pair.
[c]Total number of eggs laid per treatment.
[d]Probability that the mean number of eggs laid between a pair does not differ from the null hypothesis of 0.5.

TABLE 2. OVIPOSITION RESPONSES OF FEMALE SPICEBUSH SWALLOWTAIL BUTTERFLIES TO C18 COLUMN CHROMATOGRAPHY FRACTIONS OF AQUEOUS (POST-EtOAc) FRACTION OF SASSAFRAS LEAVES[a]

Treatment 1	$\bar{X}$[b]	Eggs[c]	Treatment 2	$\bar{X}$	Eggs	P[d]
Exp. 1. A (1.0 gle)						
Control	0.67	2	A	0.33	1	[e]
Exp. 2 B (0.5 gle) vs A + B (0.5 gle)						
Control	0.09	48.5	B	0.91	500	0.001
Control	0.08	48.5	A + B	0.92	611	0.001
B	0.46	500	A + B	0.54	611	NS
Exp. 3. A + B (1.0 gle) vs A + B + C + D (1.0 gle)						
Control	0.17	106.5	A + B	0.83	539	0.001
Control	0.18	106.5	A + B + C + D	0.82	587	0.001
A + B	0.49	539	A + B + C + D	0.51	587	NS
Exp. 4. B1 + B2 (0.25 gle) vs B (0.25 gle)						
Control	0.03	25	B1 + B2	0.97	753	0.001
Control	0.04	25	B	0.96	675	0.001
B1 + B2	0.54	753	B	0.46	675	NS
Exp. 5. B3–B6 (0.25 gle) vs B (0.25 gle)						
Control	0.14	28	B3-B6	0.86	217	0.001
Control	0.05	28	B	0.95	594	0.001
B3–B6	0.26	217	B	0.74	594	0.005
Exp. 6. Prep. B3 (0.25 gle) vs Prep. B4 (0.25 gle)						
Control	0.10	80	Prep B3	0.90	665	0.001
Control	0.13	80	Prep B4	0.87	540	0.001
Prep B3	0.56	665	Prep B4	0.44	540	NS
Exp. 7. Prep. B1 (0.25 gle) vs Prep. B2 (0.25 gle)						
Control	0.60	7.5	Prep B1	0.40	7	NS
Control	0.09	7.5	Prep B2	0.91	129	0.001
Prep B1	0.16	7	Prep B2	0.84	129	0.028
Exp. 8. Prep B5 (0.25 gle)						
Control	0.32	4.5	Prep B5	0.68	49	NS

[a]Each experiment combined the results of 6 replicated trials (N = 6–20 females/trial; NS = not significant, $P \geq 0.05$).
[b]Mean ($\bar{X}$) proportion of eggs laid on each of the two treatments in a pair.
[c]Total number of eggs laid per treatment.
[d]Probability that the mean number of eggs laid between a pair does not differ from the null hypothesis of 0.5.
[e]No statistics.

In the next series of bioassays, the B fractions were grouped as B1+B2 and B3–B6 based on HPLC patterns of peaks with similar retention times and UV spectra. B1+B2 was as active as B (Table 2, experiment 4). B3–B6 was also significantly active on its own, but less active than B (Table 2, experiment 5). The activity of these fractions was not explored further. B1 and B2 both showed a single large UV-absorbing peak on HPLC (system 1) at a retention time (R_t) of 21.9 min (compound X) in addition to other minor peaks. On TLC, this compound was revealed as a dark absorbing (λ_{254} nm) or turquoise (λ_{365} nm) spot at a R_f = 0.45. The UV spectrum (λ_{max} 235.7, 325.9) and colors on TLC indicated it was likely a hydroxycinnamic acid derivative.

Activity of Preparative B Fractions. Preparative column chromatography resulted in two B fractions, Prep. B3 and Prep. B4, that contained a large quantity of compound X. Bioassay of Prep. B3 and Prep. B4 showed both to be active alone and not significantly different from each other (Table 2, experiment 6). Prep B1 and Prep B5 were not active, but there was activity in Prep B2 (experiments 7 and 8) even though HPLC (system 1) revealed only trace amounts of compound X and other UV-absorbing compounds.

Activity of CHP20P Column Fractions of Prep B3. CHP20P subfractions of Prep. B3 were visualized on TLC to identify those containing compound X uncontaminated by other compounds. This spot was visualized alone in 30% and 35% MeOH subfractions. These were combined and labeled Bb. All other subfractions containing spots that could be visualized on TLC were combined and labeled Ba (10% to 25% MeOH subfractions). Initial bioassay results showed Ba was not active alone, although the addition of Bb to Ba resulted in oviposition activity (Table 3, experiment 1). Bb was active alone, although much more so when recombined with Ba (Table 3, experiment 2). These results suggested that two or more compounds were responsible for the activity of B.

In a second set of experiments, the later Ba column subfractions that might have contained traces of compound X were excluded. Bb again was active alone, although the addition of Ba increased oviposition activity (Table 3, experiment 3), confirming that more than one compound was responsible.

Analysis of Caffeoylquinic Acid Isomers Prepared from Chlorogenic Acid. Four major HPLC peaks at R_ts of 21.9, 30.4, 31.6, and 34.5 min, with spectra indicative of caffeoylquinic acid derivatives, as well as many minor peaks, were isomerized from chlorogenic acid. The peak at 21.9 min was isolated by column chromatography in varying degrees of purity from fractions eluting from the column in 10% MeOH in 1% aq. AcOH. Fraction F was optimal in terms of purity and concentration. The area count of the peak in fraction F was compared with the area count of the active compound X in sassafras to calculate the volume of fraction F (equivalent to 100 μg of compound X) to use in bioassay experiments.

The three other major peaks were found in the 20% MeOH fraction from

TABLE 3. OVIPOSITION RESPONSES OF FEMALE SPICEBUSH SWALLOWTAIL BUTTERFLIES TO CHP20P COLUMN CHROMATOGRAPHY FRACTIONS OF SASSAFRAS EXTRACT[a]

Treatment	$\bar{X}$[b]	Eggs[c]	Treatment 2	$\bar{X}$	Eggs	P[d]
Exp. 1. Ba (1.0 gle) vs Ba + Bb (1.0 gle)						
Control	0.28	6	Ba	0.72	21	NS
Control	0.01	6	Ba + Bb	0.99	1166	0.001
Ba	0.02	21	Ba + Bb	0.98	1166	0.001
Exp. 2. Bb (1.0 gle) vs Ba + Bb (1.0 gle)[e]						
Control	0.08	6	Bb	0.92	63	0.002
Control	0.00	6	Ba + Bb	1.00	946	0.001
Ba	0.06	63	Ba + Bb	0.94	946	0.001
Exp. 3. Bb (1.0 gle) vs Ba + Bb (1.0 gle)[f]						
Control	0.21	12	Bb	0.79	46	0.004
Control	0.04	12	Ba + Bb	0.96	301	0.001
Bb	0.13	46	Ba + Bb	0.87	301	0.002

[a]Each experiment combined the results of 6 replicated trials (N = 6–20 females/trial; NS = not significant, $P \geq 0.05$).
[b]Mean ($\bar{X}$) proportion of eggs laid on each of the two treatments in a pair.
[c]Total number of eggs laid per treatment.
[d]Probability that the mean number of eggs laid between a pair does not differ from the null hypothesis of 0.5.
[e]All fractions of Ba were combined for bioassay.
[f]Fractions of Ba uncontaminated by traces of compound X were combined for bioassay.

column chromatography. The R_f and UV spectrum (λ_{max} = 240.7, 325.9) of the peak at 31.6 min matched the retention time and UV spectrum of the standard 5-caffeoylquinic acid. The smaller peak at 34.5 min had a matching R_f and UV spectrum (λ_{max} 235.7, 320.8) of standard caffeic acid. Nagels et al. (1980) reported obtaining the 4 isomer of caffeoylquinic acid from this method and, along with others (Möller and Hermann, 1982, 1983; Brandl and Herrmann, 1983; Morishita et al., 1984; Stevenson et al., 1993) found the caffeoylquinic acid isomers eluted in the following order with HPLC C18 RP chromatography using solvent systems similar to ours: 3-, 4-, and 5-caffeoylquinic acid. This suggests that the peak at 30.4 min was the 4 isomer, although no standard was available to confirm the retention time and UV spectrum.

Five microliters of the 20% MeOH fraction, in which the HPLC peaks at 30.4 and 31.6 min corresponded closely in size to that of the active 3-CmQA peak in fraction F, were combined with Ba for bioassay.

Identification of Compound X from Sassafras. Compound X gave an UV absorption at λ_{max} 235.7 and 325.9, typical of a caffeoyl group. The low resolution FAB-MS gave a m/z at 355 (M+1) indicating a possible MF of $C_{15}H_{18}O_9$,

suggesting that compound X could be an isomer of caffeoylquinic acid. This was further confirmed by ^{1}H NMR shifts. Peaks at δ 6.19 (d, J = 16 Hz) and 7.45 (d, J = 16 Hz) (for *trans*-CH=CH—); and 6.75 (d, J = 8 Hz), 6.97 (dd, J = 8 Hz, 0.4 Hz), 7.02 (s, 1H); 9.18, (—OH); 9.57, (—OH) showed the presence of a caffeoyl moiety and peaks at δ 1.85 (m, 2H); 1.89 (m, 1H); 2.01 (m, 1H); 3.54 (m, 1H); 3.86 (m, 1H); 4.88 (m); 5.16 (m); confirmed the presence of a quinic acid moiety. The ^{1}H NMR spectrum was identical to that of 3-*trans*-caffeoyl-muco-quinic acid, an isomer of *trans*-5-caffeoylquinic acid, isolated from the foliage of *Asimina triloba* (Annonaceae) in our laboratory (Haribal et al., 1998). Its other spectral parameters and HPLC retention time also matched those of the 3-*trans*-caffeoyl-muco-quinic acid from *A. triloba*.

Synergistic Activity of CHP20P Fractions with Compound X Isolated from Sassafras Leaves and with Fraction F. Chromatography of Prep. B3 on CHP20P gel produced Ba to bioassay with compound X (100 μg) and with fraction F.

In this experiment Ba was active alone, although the addition of compound X increased the response significantly (Table 4, experiment 1). Compound X alone was not active, although the addition of Ba increased the response greatly (Table 4, experiment 2). These results showed conclusively that a minimum of two compounds were acting as oviposition stimulants, compound X and an unidentified compound(s) in Ba.

Although Ba alone was significantly more active than the control, addition of fraction F increased the response more than twofold (Table 4, experiment 3). Fraction F alone was not significantly more active than the controls (Table 4, experiment 4).

We concluded, therefore, that compound X, isolated from sassafras leaves as 3-CmQA and also found in fraction F from isomerization of chlorogenic acid, was acting as an oviposition stimulant by interaction with other compounds present in sassafras extract.

Activity of Other Chlorogenic Acid Isomers. The addition of a standard solution of 5-caffeoylquinic acid (Table 4, experiment 5), or the caffeoylquinic acid isomers present in the 20% MeOH fraction (Table 4, experiment 6), to Ba did not result in an increased oviposition response.

Amount of Active Compound X per Bean Leaf Surface Area. From 50 bean plants the following regression equation was calculated: surface area = 11.98 (wet weight) + 28.18 (F = 45.9, P = 0.001; r^2 = 0.49). This significant regression was used to estimate the amount of compound X per square millimeter of treated bean leaf surface area. By using this equation, we calculated the leaf surface areas of bean plants treated with compound X (N = 38) (mean = 144.3 ± 14.1 mm). The following formula was used to estimate the amount of compound X per square millimeter: amount = (100 μg/leaf surface area) × 10. The mean for all 38 plants treated with 100 μg of compound X was calculated as 6.99 ± 0.66 ng/mm^2.

TABLE 4. OVIPOSITION RESPONSES OF FEMALE SPICEBUSH SWALLOWTAIL BUTTERFLIES TO FRACTION Ba PLUS COMPOUND X FROM SASSAFRAS LEAVES AND TO Ba PLUS CAFFEOYLQUINIC ACID ISOMERS[a]

Treatment 1	$\bar{X}$[b]	Eggs[c]	Treatment 2	$\bar{X}$	Eggs	P[d]
Exp. 1. Ba (1.0 gle) vs Ba (1.0 gle) + compound X from sassafras leaves (100 μg)						
Control	0.17	6	Ba	0.83	47	0.02
Control	0.0	6	Ba + X	0.9	223	0.001
Ba	0.19	47	Ba + X	0.81	223	0.001
Exp. 2. Compound X (100 μg) vs compound X (100 μg) + Ba (1.0 gle)						
Control	0.42	6.5	X	0.48	16	NS
Control	0.01	6.5	Ba + X	0.99	1681	0.001
X	0.01	16	Ba + X	0.99	1681	0.001
Exp. 3. Ba (1.0 gle) vs Ba (1.0 gle) + fraction F (100 μg)						
Control	0.11	152	Ba	0.89	1300	0.001
Control	0.05	152	Ba + F	0.95	2814	0.001
Ba	0.32	1300	Ba + F	0.68	2814	0.001
Exp. 4. Fraction F (100 μg) vs Ba (1.0 gle) + fraction F (100 μg)						
Control	0.48	49	F	0.52	70	NS
Control	0.03	49	Ba + F	0.97	2175	0.001
F	0.04	70	Ba + F	0.96	2175	0.001
Exp. 5. Ba (1.0 gle) vs Ba (1.0 gle) + chlorogenic acid (5-CQA) (100 μg)						
Control	0.02	16	Ba	0.98	929	0.001
Control	0.07	16	Ba + 5-CQA	0.93	457	0.001
Ba	0.68	929	Ba + 5-CQA	0.32	457	NS
Exp. 6. Ba (1.0 gle) vs Ba (1.0 gle) + 20% MeOH fraction (5 μl)						
Control	0.07	36	Ba	0.93	562	0.001
Control	0.21	36	Ba + 20% fr.	0.79	672	NS
Ba	0.51	562	Ba + 20% fr.	0.49	672	NS

[a]Each experiment combined the results of 6 replicated trials (N = 6–20 females/trial; NS = not significant; $P \geq 0.05$).
[b]Mean ($\bar{X}$) proportion of eggs laid on each of the two treatments in a pair.
[c]Total number of eggs laid per treatment.
[d]Probability that the mean number of eggs laid between a pair does not differ from the null hypothesis of 0.5.

DISCUSSION

3-*trans*-Caffeoyl-muco-quinic acid is the first oviposition stimulant reported for the spicebush swallowtail butterfly. This compound is not active alone, but it synergizes the activity of one or more other compounds, as yet unidentified, to increase oviposition responses. This is also the first report of a swallowtail oviposition stimulant isolated from a plant in the family Lauraceae.

In the butterfly family Papilionidae, hydroxycinnamic acid derivatives are known as oviposition stimulants for two other swallowtail species in the genus *Papilio* of the tribe Papilionini. *trans*-5-Caffeoylquinic acid has been identified as an oviposition stimulant for the black swallowtail butterfly, *P. polyxenes*, from two of its hosts, wild carrot, *Daucus carota* (Feeny et al., 1983, 1988), and wild parsnip, *Pastinaca sativa* (Carter et al., 1998). In his investigation of the *Citrus*-feeding *P. protenor*, Honda (1990) found that *trans*-5-caffeoylquinic acid was one of several oviposition stimulants isolated from the leaves of rutaceous host plants. Hydroxycinnamic acid derivatives have also been identified as oviposition stimulants for swallowtails in two other tribes, the Graphiini and Troidini. In the tribe Graphiini, *Eurytides marcellus*, the zebra swallowtail, uses 3-CmQA as the major contact oviposition stimulant from its host plant, *A. triloba*, in the plant family Annonaceae (Haribal et al., 1998). *Parides* (*Atrophaneura*) *alcinous* in the tribe Troidini uses the compound 3-hydroxy-4-methoxycinnamoylmalic acid as one of the oviposition stimulants from its host plant *Aristolochia debilis* (Nishida, 1995).

Host association patterns of swallowtails seen today are believed to be a result of repeated colonizations of plants belonging to a small number of families, some distantly related, subsequent to plant cladogenesis (Miller, 1987). Most swallowtail species are specialists using only members of one plant family as hosts (Scriber, 1984; Scriber et al., 1991; Feeny, 1995). Approximately 80% of swallowtail species with existing host records are recorded from the plant families Lauraceae, Annonaceae, Aristolochiaceae, Rutaceae, and Apiaceae (Umbelliferae) (Feeny, 1991; Scriber et al., 1991). As detailed above, hydroxycinnamic acid derivatives have now been isolated as oviposition stimulants from all five of these families, providing one phytochemical link between plant families used as hosts by swallowtail specialists. From results reported so far, a 3-isomer of chlorogenic acid is used by swallowtails specializing on plants in the Annonaceae and Lauraceae, two families more closely related to each other than to plants in the Rutaceae and Apiaceae (Cronquist, 1981; Miller, 1987; Thorne, 1992) where the 5 isomer acts as an oviposition stimulant. If the distribution of these isomers is limited in host-plant families, swallowtails could have evolved responses to particular isomers found in association with other host-specific chemical, visual, volatile, or physical host plant cues.

The results presented here lend support to the hypothesis of behavioral facilitation put forth by Dethier (1941) originally to explain swallowtail feeding behavior and argued more recently by Feeny (1991, 1995) in terms of oviposition chemistry. The hypothesis states that chemical similarities among hosts of related insect species result from preadaptation by the insects in their responses to behavioral cues (Feeny, 1991). Preadaptation to oviposit in response to hydroxycinnamic acid derivatives could be one of the chemical similarities likely to have facilitated host-plant colonizations by some swallowtail species.

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INDUCTION AND SYSTEMIC RELEASE OF HERBIVORE-INDUCED PLANT VOLATILES MEDIATING IN-FLIGHT ORIENTATION OF *Aphidius ervi*

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Abstract—In-flight orientation of the braconid *Aphidius ervi* in response to volatiles released from broad bean plants infested by the pea aphid, *Acyrthosiphon pisum*, was studied in a no-choice wind-tunnel bioassay. The role of aphid infestation level and duration, systemic production of volatiles by "insect-free" parts of the plant, and the specificity of aphid-induced volatiles on the flight behavior of the foraging female parasitoids were investigated. The upper insect-free part of a three-leaved broad bean plant, which was basally infested by a population of 40 *A. pisum*, released synomones detectable by *A. ervi* females after at least 48–72 hr of infestation, resulting in both significant increases in oriented flights and landings on the source compared with uninfested control plants. This suggests that volatiles involved in host-location by *A. ervi* are systemically released by broad bean plants either in response to circulation of aphid saliva, circulation of saliva-induced bioactive elicitors, or circulation of the synomones themselves. Air entrainment extracts of volatiles collected from a broad bean plant infested by the nonhost *Aphis fabae* or an uninfested broad bean plant elicited few oriented flights and landing responses by female parasitoids. These extracts were significantly less attractive than extracts collected from a broad bean plant infested by the host *A. pisum*, indi-

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cating the specificity of synomones elicited by different aphid species on the same plant species.

Key Words—Host location, plant volatiles, aphid feeding, wind tunnel, *Acyrthosiphon pisum*, *Aphis fabae*, air entrainment, tritrophic interactions.

INTRODUCTION

Long-range chemical cues play a crucial role in host location by foraging female parasitoids (Vinson, 1985, 1991). In the initial steps of the foraging process, parasitoid response to herbivore-induced plant volatiles appears to offer a solution to the reliability–detectability problem, as hypothesized by Vet and Dicke (1992). These cues are both highly detectable and reliable since they are produced in large amounts by the plant and only as a consequence of a herbivore attack (Dicke et al., 1990a, 1993; Turlings et al., 1990, 1991; Takabayashi et al., 1991, 1994, 1995; Steinberg et al., 1993; Dicke, 1994; Geervliet et al., 1994). Previous studies have demonstrated that the release of these attractive herbivore-induced volatiles does not exclusively occur from infested organs, but is often a systemic response (Dicke et al., 1990b; Turlings and Tumlinson, 1992) induced by elicitor(s) systemically translocated throughout the plant (Dicke et al., 1993; Turlings et al., 1993; Alborn et al., 1997). Even uninfested plants can be stimulated to produce and release attractive herbivore-induced volatiles by treating them with herbivore regurgitant (Turlings et al., 1993; Mattiacci et al., 1994; Alborn et al., 1997). Similarly, uninfested bean leaves placed in water that had previously been in contact with mite-infested leaves, became attractive to predatory mites (Dicke et al., 1993). In some cases, the process that leads to the alteration of plant volatile profiles seems to be dependent on both the size of the herbivore infestation levels and the duration of feeding activity (Dicke et al., 1993; Tumlinson et al., 1993).

Recently, the role of volatiles from first and second trophic levels that regulate the foraging behavior of *Aphidius ervi* Haliday has been elucidated by using both Y-tube olfactometers and wind tunnels (Wickremasinghe and Van Emden, 1992; Guerrieri et al., 1993, 1996; Du et al., 1996; Poppy et al., 1997a). This parasitoid is reported as an effective biological control agent of both the pea aphid, *Acyrthosiphon pisum* (Harris), and cereal aphids (Powell, 1982; Pennacchio, 1990) and is becoming a model aphid parasitoid species for behavioral studies (Battaglia et al., 1993, 1995; Pennacchio et al., 1994; Du et al., 1997; Guerrieri et al., 1997; Poppy et al., 1997b; Powell et al., 1998).

In both choice and no-choice wind-tunnel bioassays, both the plant host complex (broad bean *Vicia faba–A. pisum*) and host-damaged plants from which aphids had been removed elicited similar percentages of oriented flights and were

significantly more attractive to female *A. ervi* than uninfested plants, mechanically damaged plants, or host aphids by themselves (Guerrieri et al., 1993; Du et al., 1996). These results led to the hypothesis that there are herbivore-induced synomones produced by the plant that *A. ervi* females exploit during host location. Furthermore, the changes in volatile profile induced by aphid feeding appeared to be species-specific: the parasitoid was able to discriminate between the plant–host complex (broad bean plants infested by *A. pisum*) and a plant–nonhost complex (broad bean plants infested by the nonhost *Aphis fabae* Scopoli) in both choice and no-choice wind-tunnel bioassays (Du et al., 1996).

In the present study, we investigated the origin, specificity, and release of herbivore-induced synomones. With a wind-tunnel bioassay, we examined the change in attractiveness over time of plant–host complexes comprising different *A. pisum* infestation levels and/or differing durations of feeding. This was done in order to assess the minimal infestation level/time combination necessary to make the plant attractive to the parasitoid. The possible systemic release of herbivore-induced synomones by plants infested with aphids was also investigated by evaluating the attractiveness of parts of the plant not infested with aphids. Air entrainment extracts were used to verify that plant volatiles are at least partially responsible for eliciting the observed behavioral responses of *A. ervi*.

METHODS AND MATERIALS

Experimental Organisms

Experiments were carried out in Italy and the UK with two different parasitoid strains, referred to below as Italian and British strains, respectively. *A. ervi* was reared on its natural host *A. pisum* and maintained on potted broad bean (*Vicia faba*) plants, cv. Aguadulce (Italian variety) and Sutton (British variety). Aphid and parasitoid cultures were kept in separate environmental chambers at $20 \pm 1°C$ (Italian strain) and $18 \pm 1°C$ (British strain), $75 \pm 5\%$ relative humidity, and 18L : 6D photoperiod. The nonhost black bean aphid (*A. fabae*) was also reared on *V. faba* cv. Sutton at $18 \pm 1°C$, $75 \pm 5\%$ relative humidity, and 18L : 6D photoperiod.

Insect parasitoids used in the bioassays were reared as synchronized cohorts by exposing heavily infested plants for 24 hr to 1-day-old mated females; after a week, the resultant mummies were clipped from the plant and isolated in glass test tubes (60×8 mm) plugged with cotton wool. Experimental females were used between the first and second day after emergence, mated, and fed with a 50% honey solution. All experiments were conducted 3–7 hr from the onset of photophase.

Wind Tunnels

Experiments with the Italian strain were carried out in a wind tunnel similar to that described by Miller and Roelofs (1978), modified according to Guerrieri et al. (1993). Experiments with the British strain were carried out in a similar tunnel as described by Du et al. (1996). Previous studies confirmed that these two wind tunnels give comparable results when assessing the foraging behavior of *A. ervi* (Guerrieri et al., 1993; Du et al., 1996).

The wind speed, monitored with a hot-wire anemometer (Alnor, model CGA-26), was adjusted to 20 ± 1 cm/sec in all experiments. The exhausted air was vented outside the wind-tunnel rooms. The light intensity in the flight chambers was around 3600 lux at the take off point. A black and white striped pattern was placed beneath the flight chamber floors to aid visual orientation.

Entrainment of Plant Volatiles

All glassware, including the air entrainment equipment, was cleaned and heated at 200–230°C overnight before use. Solvents were purified by redistillation. Porapak Q (50–80 mesh) was used to collect volatiles in air entrainment experiments; it was washed with redistilled diethyl ether and then heat conditioned overnight by a nitrogen stream at 180°C. A 3-cm length of Porapak (50 mg) was placed inside a small glass tube and held between two plugs of silanized glass wool.

Bean plants (cv. Sutton) were transferred from plastic pots to glass Pyrex beakers three to four days before starting an experiment. All experiments were started with plants having three pairs of leaves fully developed (approximately 14-day-old plants). Glass beakers, each containing two plants, were separately placed into a 5-liter Quickfit FV Culture Vessel (Phillip Harris Scientific) entrainment apparatus. The system we used was designed after Blight (1990) and allowed three lines to be run simultaneously.

Air was purified by passing through molecular sieve (5Å) and activated charcoal before entering the fermentation vessels. Airflow was adjusted to 1 liter/min. Collected volatiles were adsorbed onto Porapak Q and eluted with freshly distilled diethyl ether (500 μl). The elutant was brought up to a volume of 500 μl by using redistilled diethyl ether and stored in Teflon-capped vials when tested shortly after collection, or sealed in an ampoule under nitrogen (N_2) when tested later. All samples were stored in a freezer at −20°C until use in the bioassay.

Bioassay

Experiments were carried out at 20 ± 1°C and 65 ± 5% relative humidity. Individual parasitoids were used once and released from an open-ended glass test

tube held on a plastic base so that the take-off point was exactly in the middle of a cross section of the flight chamber. Between 40 and 80 parasitoid females were tested for each target by releasing them individually in the odor plume, 20 cm downwind from the target. Parasitoids were observed for a maximum time of 10 min, and flight behavior data were recorded and analyzed with the aid of event-recording software (The Observer, Noldus Information Technology, Wageningen, The Netherlands.) A flight response was considered as oriented towards the experimental target when the female flew directly upwind and landed on or not further than 5 cm away from the target. Experiments were conducted on several days, and targets were presented in a random order to reduce the effect of uncontrolled temporal variability of behavioral responses.

Experiment I: Aphid Density and Time After Initial Infestation

This experiment was designed to assess the systemic release of herbivore-induced synomones from uninfested parts of the plant and the minimum number of aphids/hours (i.e., the number of aphids feeding for varying time durations) required to make the plant attractive to *A. ervi* foraging females.

A broad bean plant with three fully developed leaves was infested with mixed instars of *A. pisum*, which were confined to the basal leaf in a small circular plastic clip cage (6 cm diameter), similar to those described by Mackauer and Bisdee (1965). After 72 hr, the infested leaf was removed, the wound covered with parafilm, and the upper part of the plant immediately used as a target in the wind tunnel. Three different infestation levels were used: 10, 20, and 40 aphids per plant. An uninfested plant, with its basal leaf removed in the same way, was used as a control. The minimal infestation level that elicited a significant increase in parasitoid response was then used in similar experiments of shorter duration (24 and 48 hr) to determine the role of feeding duration on plant attractiveness.

Experiment II: Responses to Volatiles Collected as Entrainment Extracts

This experiment was designed to assess whether the volatiles collected from plants that had been infested for varying time durations elicited a flight response in the same way as the infested plants. This was done also to define more precisely the critical time interval from the onset of infestation that is required to induce plant attractiveness. Two broad bean plants with three fully developed leaves were uniformly infested with 40 *A. pisum* of mixed instars each and entrainment extracts of volatiles were collected over 24-hr time intervals for five consecutive days.

Extracts were placed onto small pieces (3 × 1 cm) of filter paper (Whatman No. 1), which were inserted into an open-ended glass tube (6 × 1 cm) with the end facing the parasitoid surrounded by a green circular target (8 cm diameter).

This was done to mimic the visual cues normally provided by the plant. Preliminary trials indicated that a test concentration of 2.2 hr equivalent was sufficient to induce parasitoid flight orientation. This concentration was used in all tests, and fresh extract aliquots were used for each experimental female.

Experiment III: Attraction to Systemically Released Volatiles

This experiment was designed to assess whether we could extract the herbivore-induced synomones that are systemically released from the undamaged parts of the plant and still get the response observed in experiment I.

Air entrainment extracts of the volatiles were collected from two three-leaved broad bean plants, each infested for 72 hr by 40 *A. pisum* of mixed instars confined on the basal leaf as described above. This infested leaf was removed, the wound covered with parafilm, and the upper part of the plant used to collect volatiles extracts over 24 hr (host damaged plant– systemically, HDP-SYS). An uninfested three-leaved plant with its basal leaf removed in the same way was used to obtain control entrainment extracts (undamaged plant—systemically, CONTR-SYS). All extracts were bioassayed in the wind tunnel according to the methodology described for Experiment II.

Experiment IV: Attraction to Volatiles from Plants Infested by Hosts and Nonhosts

The attractiveness of plant volatiles induced by host and nonhost aphid species was compared. Extracts were collected over a 55-hr time interval from broad bean plants (cv. Sutton) standardized as above and uniformly infested for at least three days by about 150 *A. pisum* (British strain) (plant host complex, PHC) or *A. fabae* (plant nonhost complex, PNHC); the volatiles from uninfested plants (cv. Sutton) (uninfested plant, P) acted as a control. All extracts were bioassayed in the wind tunnel according to the methodology described for experiment II.

Statistical Analyses

The daily mean percentage responses to each target type in experiments I, II, and IV were calculated. The proportions responding (i.e., showing oriented flights and landings on the target) were subjected to logit transformation to normalize the data and then analyzed by using one-way analysis of variance to test the effect of the target treatment on the response. Tukey multiple comparison tests were used to compare individual means.

The number of parasitoids responding to each target of experiment III were compared by a χ^2 test.

RESULTS

Experiment I: Aphid Density and Time After Initial Infestation

The number of aphids feeding for 72 hr affected the attractiveness of the plant to female parasitoids both in terms of oriented flight (F = 44.91, d.f. 3,5, $P < 0.001$) and landing (F = 12.19, d.f. 3,5, $P < 0.01$) responses. Parasitoid responses to a broad bean plant infested for 72 hr by 10 or 20 aphids were not significantly different from those observed for uninfested plants (Figure 1). In contrast, when plants had been infested by 40 pea aphids for 72 hr they became significantly more attractive than the control ($P < 0.05$), both in terms of oriented flights and landings on the target source (Figure 1).

The duration of feeding by the 40 aphids affected the attractiveness of the plant to female parasitoids, both in terms of oriented flight (F = 5.27, d.f. 3,6, $P < 0.05$) and landing (F = 6.26, d.f. 3,6, P = 0.05) responses. The attractiveness of

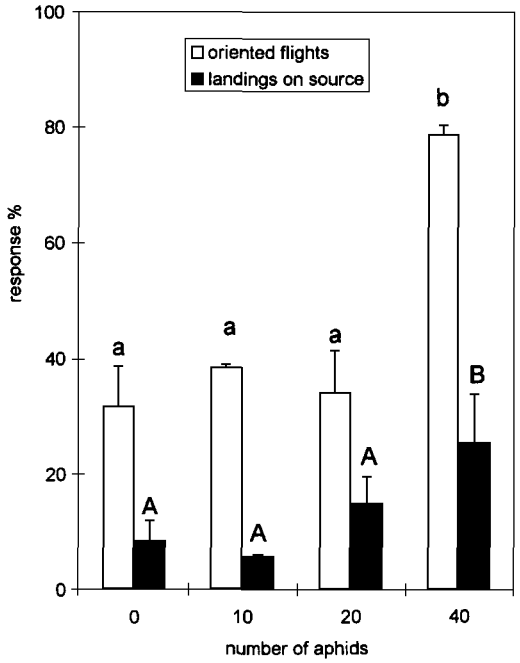

FIG. 1. Percentages of *Aphidius ervi* females responding to broad bean plants basally infested for 72 hr by a different number of mixed instars of *Acyrthosiphon pisum*. Flight (lowercase letters) and landing (uppercase letters) responses with different letters are significantly different ($P < 0.05$), based on Tukey multiple comparison tests.

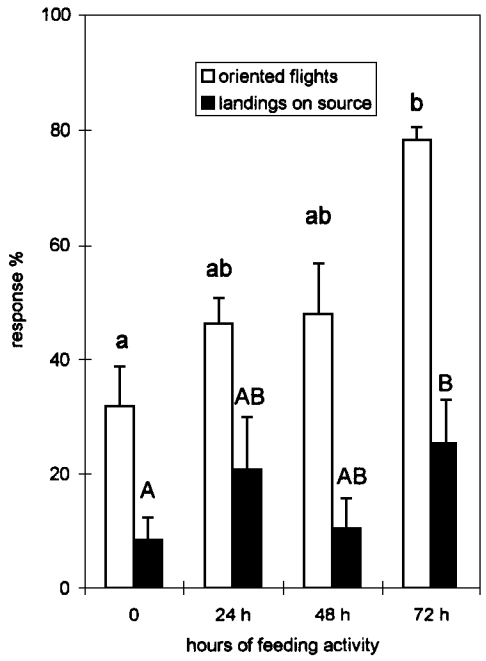

FIG. 2. Percentages of *Aphidius ervi* females responding to broad bean plants basally infested by 40 *Acyrthosiphon pisum* of mixed instars feeding for different numbers of hours. Flight (lowercase letters) and landing (uppercase letters) responses with different letters are significantly different ($P < 0.05$), based on Tukey multiple comparison tests.

broad bean plants appeared to increase as the duration of feeding increased (Figure 2). However, for a population of 40 pea aphids, 72 hr of feeding was required to increase the number of oriented flights or landings by *A. ervi* females ($P < 0.05$), compared to a control plant.

Experiment II: Responses to Volatiles Collected as Entrainment Extracts

There was a difference between the volatile entrainment extracts collected at different time intervals both in terms of oriented flight ($F = 15.13$, d.f. 4,17, $P < 0.001$) and landing ($F = 7.26$, d.f. 4,17, $P < 0.01$) responses. Volatiles collected during the time intervals 0–24 and 24-48 hr from the beginning of infestation elicited significantly fewer oriented flights ($P < 0.05$) than the volatile extracts collected at 48-72, 72-96, and 96-120 hr (Figure 3). When considering landings on the volatile source, a similar pattern was observed, with more landings recorded for volatiles collected over 48–72 hr and 96–120 hr compared with the first time interval 0–24 hr ($P < 0.05$) (Figure 3).

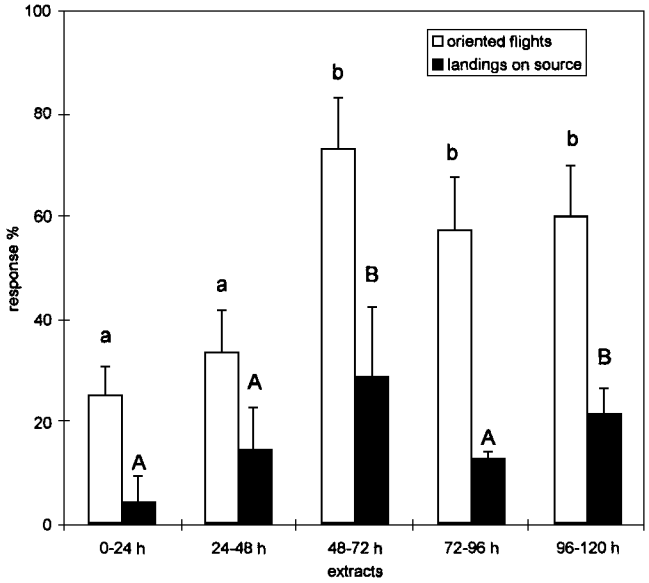

FIG. 3. Percentages of *Aphidius ervi* females responding to air entrainment extracts of volatiles collected at different time course intervals from two broad bean plants, each uniformly infested by 40 *Acyrthosiphon pisum* of mixed instars. Flight (lowercase letters) and landing (uppercase letters) responses with different letters are significantly different ($P < 0.05$), based on Tukey multiple comparison tests.

Experiment III: Attraction to Systemically Released Volatiles

Volatiles collected from the aphid-free part of broad bean plants that had been infested only on the basal leaves (HDP-SYS) elicited more oriented flights by *A. ervi* females than did control plants (CONTR-SYS) ($\chi^2 = 4.01$, d.f. 1, $P < 0.05$), although landings on the source were not significantly different (Figure 4).

Experiment IV: Attraction to Volatiles from Plants Infested by Hosts and Nonhosts

Volatiles collected from PNHC and P were significantly less attractive to *A. ervi* females than those from PHC ($P < 0.05$), eliciting fewer oriented flights (Figure 5), but no differences were observed in terms of landings.

DISCUSSION

The results demonstrate that 40 *A. pisum* of various instars feeding for at least 60–72 hr alters the secondary metabolism of a young broad bean plant,

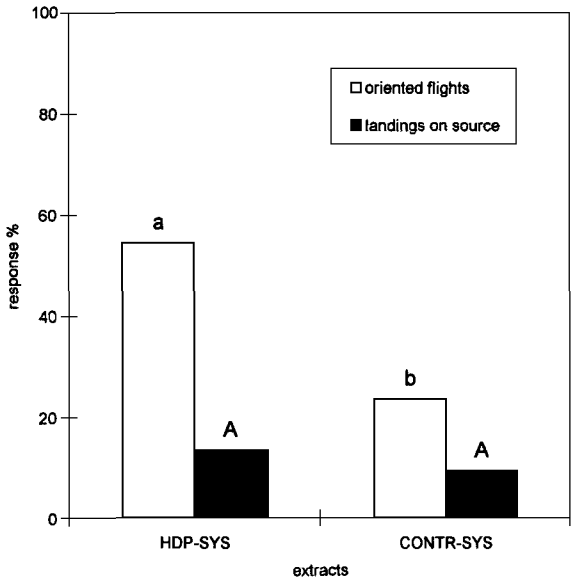

FIG. 4. Percentages of *Aphidius ervi* females responding to air entrainment extracts of volatiles collected for 24 hr from 2 broad bean plants previously infested for 72 hr by 40 *Acyrthosiphon pisum* of mixed instars confined on a basal leaf, which was removed before starting volatile collection (HDP-SYS), and from two uninfested broad bean plants from which the basal leaf was similarly removed before starting volatile collection (CONTR-SYS). Flight (lowercase letters) and landing (uppercase letters) responses with different letters are significantly different ($P < 0.04$), based on χ^2 comparison tests.

leading to the production of synomones that attract foraging *A. ervi* females. The induction of synomones in broad bean plants as a result of aphid feeding has been previously demonstrated by Guerrieri et al. (1993) and Du et al. (1996), but neither of these studies investigated the effect of infestation level and duration on attractiveness. The time required before the plant becomes attractive to parasitoids corresponds to the temporal production of induced volatiles by aphid infested broad bean plants (see Du et al., 1998).

De novo synthesis of several compounds that are attractive to parasitoids, activated by one or more herbivore-derived elicitors, has been reported for cotton plants (Paré and Tumlinson, 1997). Similarly, corn plants start to release compounds that are attractive to *Cotesia marginiventris* (Cresson) females several hours after the initial injury by their lepidopteran host *Spodoptera exigua* (Hübner) (Turlings et al., 1990). The apparently longer time interval needed to induce the aphid-infested plant to start producing or release enough compounds attractive to *A. ervi* could be due to the less mechanically disruptive feeding

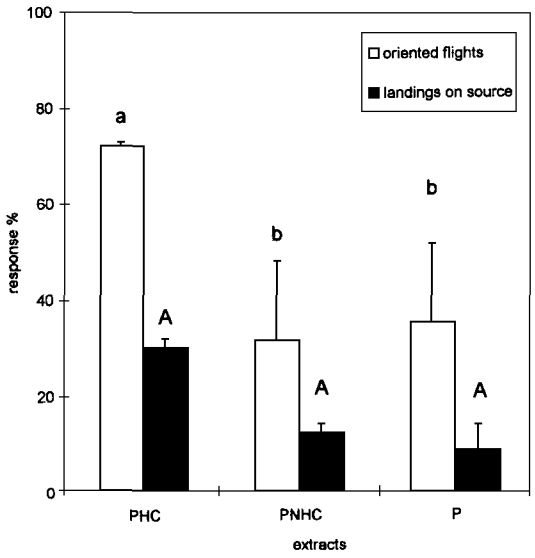

FIG. 5. Percentages of *Aphidius ervi* females responding to air entrainment extracts of volatiles collected for 55 hr from two broad bean plants uniformly infested by hundreds of the host aphid *Acyrthosiphon pisum* (PHC), two broad bean plants uniformly infested by hundreds of the nonhost aphid *Aphis fabae* (PNHC), and two uninfested broad bean plants (P). Flight (lowercase letters) and landing (uppercase letters) responses with different letters are significantly different ($P < 0.001$), based on Tukey multiple comparison tests.

habit of sucking aphids compared with chewing lepidopteran larvae, or it could be interpreted as a parameter linked directly with infestation density. Further studies are needed to address this question. However, we hypothesize that aphid saliva injection induces significant changes to the plant secondary metabolism, leading to quantitative and/or qualitative changes to volatile profiles.

Our results also demonstrate that the production of aphid-induced synomones is not restricted to infested leaves, but occurs systemically throughout the plant. The upper part of a broad bean plant that was infested with aphids only on the basal leaf (removed prior to experimentation), was attractive to foraging female parasitoids. This would lend further support to our hypothesis that salivary secretions injected while feeding are involved in systemic changes in the production of plant volatiles.

Further studies are needed to determine whether plant volatiles attractive to *A. ervi* are translocated from the feeding site throughout the rest of the plant, or are de novo synthesized in response to translocation of the saliva or a saliva-induced bioactive elicitor. In another tritrophic system, the translocation of an endogenous and water-soluble elicitor has been demonstrated (Dicke et

al., 1993). Uninfested and undamaged Lima bean leaves initiated the release of synomones attractive to a predatory mite, when incubated in water in which spider mite-infested leaves had been present for the previous seven days (Dicke et al., 1993). The existence of exogenous elicitors also has been demonstrated in other cases. By inoculating stems of an uninfested corn plant with the regurgitant of *Chilo partellus* (Swinhoe) feeding larva, leaves were stimulated to produce herbivore-induced synomones that attracted *Cotesia flavipes* Cameron females (Potting et al., 1995). Mechanically damaged corn plants treated with *Spodoptera exigua* regurgitant, as well as undamaged seedlings incubated in an aqueous solution of caterpillar regurgitant, increased production of terpenes attractive to both the generalist *Cotesia marginiventris* and the specialist *Microplitis croceipes* Cresson (Turlings et al., 1993). *Cotesia glomerata* (L.) also reacted positively to mechanically damaged Brussels sprout plants when treated with *Pieris brassicae* L. regurgitant (Mattiacci et al., 1994).

The different gas-chromatography profiles of the air entrainment extracts from host and nonhost complexes (Guerrieri et al., 1996; Du et al., 1998), as well as the differential attraction of *A. ervi* females to such extracts demonstrated in this work, support the results obtained by Du et al. (1996) that *A. ervi* can distinguish between plants infested with host or nonhost aphids. These findings indicate that metabolic alteration of the plant differs both quantitatively and qualitatively according to the host aphid species feeding on it (Du et al., 1998; Powell et al., 1998). Similar degrees of specificity at the herbivore level have been reported in only a few of the tritrophic systems investigated so far. The predatory mites *Amblyseius potentillae* (Garman) and *A. finlandicus* (Oudemans) are attracted by apple leaves infested by their prey the phytophagous mite *Panonychus ulmi* (Koch), but not by the unsuitable prey *Tetranychus urticae* Koch (Sabelis and Van de Baan, 1983). These differing responses appear to be due to the different ratios of compounds in the volatile mixtures released by the infested plants (Takabayashi et al., 1991). In contrast, naive *Cotesia rubecula* (Marshall) (specialist on *Pieris rapae* L.) and *Cotesia glomerata* (generalist on *Pieris* spp. and *Aporia crataegi* L.) do not discriminate between host- and nonhost-damaged plants even though they may show a preference for certain cabbage cultivars and plant species (Geervliet et al., 1996). Similarly, both the generalist parasitoid *C. marginiventris* and the specialist *M. croceipes* were unable to discriminate between plants treated with the regurgitant of several species of caterpillars (hosts and nonhosts) as well as that of a grasshopper (Turlings et al., 1993).

It appears, therefore, that a complex interaction occurs between aphids and plants leading to the production of herbivore-induced synomone blends that differ depending on the aphid species involved. Further studies are currently underway investigating the nature and the role of the elicitor in aphid saliva and the behavioral responses of aphid parasitoids representing different degrees of host specialization.

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ISOLATION AND IDENTIFICATION OF PRIMARY METABOLITE FEEDING STIMULANTS FOR ADULT WESTERN CORN ROOTWORM, *Diabrotica virgifera virgifera* LECONTE, FROM HOST POLLENS

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Abstract—Consumption of pollens from several host plants was quantified for adult western corn rootworm, *Diabrotica virgifera virgifera*, with significantly more sweet corn and winter squash pollen consumed than that of common sunflower and Canada goldenrod. Sequential solvent extraction and chromatographic fractionation of host pollens, guided by a feeding bioassay of respective fractions, isolated dominant phagostimulatory activity in pools of free amino acids. Amino acid profiles were determined by TLC and HPLC analysis. For sweet corn pollen, proline was predominant, accounting for 70% of a total 95.3 μmol free amino acid/g pollen; serine, alanine, γ-aminobutyric acid (GABA), and asparagine occurred in decreasing order of concentration. In squash pollen, β-alanine predominated (30% of 111 μmol amino acid/g pollen), followed by asparagine, alanine, proline, and GABA. Histidine (42% of 83.8 μmol amino acid/g pollen), proline, aspartic acid, asparagine, and alanine were dominant for sunflower pollen; and for goldenrod, proline (51% of 50.6 μmol amino acid/g pollen), *trans*-hydroxyproline, serine, histidine, and alanine predominated. The five dominant amino acids from each pollen accounted for 80–90% of total amino acid content. Simple mixtures of some dominant amino acids, approximating levels found in sweet corn and squash pollen, gave phagostimulation similar to that of the crude extracts.

Key Words—Amino acid, phagostimulant, host association, pollen feeding, Chrysomelidae, Diabroticite.

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INTRODUCTION

The mechanisms driving host associations in phytophagous insects, particularly the effects of plant chemistry on insect herbivores, have long been debated (Dethier, 1954; Fraenkel, 1959). Initially, the discussion centered on the importance of primary (Thorsteinson, 1960; Beck, 1965) versus secondary (Dethier, 1954; Fraenkel, 1959, 1969) plant metabolites in determining food preference. Since the study of Ehrlich and Raven (1964), most attention has been given to secondary metabolites as attractants, feeding deterrents, or toxicants for phytophagous insects. Primary metabolites such as sugars (Bernays and Chapman, 1994) and amino acids (Hollister and Mullin, 1998) have repeatedly been shown to be phagostimulants for many insects, but the role of primary plant metabolites in determining host preferences or acceptability has become overshadowed by this focus on secondary chemistry (Berenbaum, 1995). Here, we identify amino acids, not carbohydrates, as dominant phagostimulatory cues in the association of the western corn rootworm *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) with host plant pollens.

Pollen feeding occurs in many orders of insects, including Coleoptera (Ludwig and Hill, 1975; Jones et al., 1993; Jayanth et al., 1993), Diptera (Hickman et al., 1995; Wacht et al., 1996), Thysanoptera (Kirk, 1985), Lepidoptera (Gilbert, 1972), and Hymenoptera (Dobson and Peng, 1997). For chrysomelid beetles, association with pollen is thought to have occurred early in their evolution, and many are described as being at least facultative pollen feeders (Samuelson, 1994). Despite this association, and observations of adult diabroticite consumption of pollen (Ludwig and Hill, 1975; Siegfried and Mullin, 1990; Samuelson, 1994), little is known of this interaction. Here, we present the first quantitative investigation of *Diabrotica* pollen consumption behavor.

The chemical basis and mechanisms underlying pollen use in insects have been investigated in a few cases. Delisle et al. (1989) observed an ethanolic extract of sunflower pollen to be an oviposition stimulant for the sunflower moth, *Homoeosoma electellum* (Hulst). An aqueous extract of *Parthenium* pollen was shown by Jayanth et al. (1993) to stimulate consumption by the chrysomelid beetle, *Zygogramma bicolorata* Pallister, when smeared on sunflower leaves. The influence of chemical stimuli on the control of pollen ingestion in *Eristalis tenax* L. was investigated by Wacht et al. (1996). They found that water-washed pollen was consumed less than untreated pollen and that a distilled water extract stimulated activity in labellar taste hairs. In these studies, however, the chemicals eliciting behavior were not identified. Here, we report the isolation and identification of pools of free amino acids as dominant feeding stimulants in host pollens for adult *Diabrotica*.

METHODS AND MATERIALS

Insects. Field-collected and a nondiapausing strain of adult western corn rootworm (WCR) (French Agricultural Research, Inc., Lamberton, Minnesota) were used for behavioral assays. Nondiapausing insects were received as pupae and kept in moistened soil in an environmental chamber until emergence. Newly emerged beetles were moved to plastic boxes and maintained on acorn squash and water. Ten- to 18-day-old beetles were starved 24 hr prior to use in feeding bioassays.

Adult WCR of unknown age were collected from winter squash var. Blue Hubbard (*Cucurbita maxima* Duchesne) plants in a multicrop field plot (0.4 ha) at The Russell Larson Field Research Center at Rock Springs in Centre County, Pennsylvania. Insects were collected in the field, put on ice, and transported to the laboratory where species of diabroticites were separated. Adult WCR were placed in plastic boxes and maintained on acorn squash and water. Beetles were starved 24 hr prior to use in feeding bioassays.

Pollen. Intact flowers including upper stems of sweet corn (*Zea mays* L.) var. Bodacious and Giant Gray Stripe sunflower (*Helianthus annuus* L.) were collected a few hours prior to dehiscence from the multicrop field plot at Rock Springs used for beetle collection. Canada goldenrod (*Solidago canadensis* L.) was obtained from adjacent meadows. Inflorescences were cut with about 30 cm of stem, returned to the laboratory on ice, placed in water, and allowed to shed pollen into aluminum trays. Winter squash var. Blue Hubbard pollen was collected from blossoms of greenhouse plants grown in Metro-Mix 200 (Terra-Lite) under halogen lights (16L : 8D). Pollen was sieved through 243- (squash), 153- (sween corn), and 80-μm (sunflower and goldenrod) nylon screens (Tetko Inc., Briarcliff Manor, New York) and stored below $-20°C$ until use.

Chemical Extract. Sequential surface extraction of 1.5 g pollen on a sintered glass filter funnel or inert filter membrane was done with petroleum ether (3×10 ml), methanol (3×10 ml), and water (3×4 ml). After each solvent addition, pollen was periodically agitated for 3 min prior to vacuum filtration. Extracts were evaporated on a Buchi rotovapor under reduced pressure and resuspended in the original solvent for bioassay. Combined methanol–water extracts were further processed through 1.5-g Dowex 50X8 cation-exchange resin (H^+ form) packed into a 21-cm Pasteur pipet. The column was washed with 5 ml water and bound cations were then eluted with 10 ml 1 : 7.5 15 N NH_4OH–H_2O; the original alcoholic water, cation-exchange water wash, and NH_4OH eluate fractions were separately evaporated to dryness and resuspended in water for bioassay after adjustment of pH to 6.5–7.0.

Extract Analysis. Extracts were analyzed for free amino acids and carbo-

hydrates using 20-cm-long analytical thin-layer chromatography (TLC) plates (E. Merck Kieselgel 60 F-254). For free amino acids, a single-step solvent development system was used: ethyl acetate–methanol–water (50 : 40 : 20). Ninhydrin spray reagent (2 mg/ml 100% ethanol) followed by heating was used to visualize free amino acids, while naphthoresorcinol (2 mg/ml in 8.7 parts 100% ethanol–1.3 parts 85% phosphoric acid) and heating to 100°C detected the carbohydrates (Stahl, 1967). Ultraviolet-absorbing compounds were visualized as a quench of background fluorescence at 254 nm. Further TLC analyses of amino acids in sweet corn, squash, and sunflower methanol extracts and in ion-exchange ammonium hydroxide fractions were done using phenylthiohydantoin (PTH) amino acid derivatives [adapted from Bidlingmeyer et al. (1984) and Edman (1950)]. Protein and nonprotein amino acid standards (Sigma 99+%, all L isomers unless noted otherwise) or extract fractions were first derivatized with phenylisothiocyanate (PITC). Samples were evaporated to dryness, mixed with a CH_3OH–H_2O–triethylamine (TEA, 2 : 2 : 1) solution, dried, then mixed with a CH_3OH–H_2O–TEA–PITC (7 : 1 : 1 : 1) solution, and left to react for 30 min in a 26°C water bath. PITC–amino acid derivatives were then cyclized to the respective PTH by treatment with 3.3 M HCl at 60°C for 15 min. PTH derivatives were extracted from the aqueous mix with ethyl acetate, concentrated, and resolved by TLC with a couple of two-step solvent systems: (1) to 6 cm with chloroform–formic acid (100 : 5), then full development with chloroform–methanol (97 : 3); and (2) to 6 cm with chloroform–methanol–formic acid (90 : 10 : 1), then full development with chloroform–ethyl acetate–methanol (96 : 3 : 1). PTH–amino acids were visualized by UV quench at 254 nm.

Extracts were analyzed for the presence of quaternary ammonium compounds by TLC in isopropanol–H_2O (60 : 30). Compounds were visualized with Dragendorff's reagent (Stahl, 1967; Storey and Wyn Jones, 1977). Phenolics, flavonoids, and carotenoids were detected spectrally on TLC plates with a Shimadzu Flying Spot Dual Wavelength Scanning Densitometer or after elution from the plates using a Perkin-Elmer Lambda 3β UV/Vis spectrophotometer.

Free amino acids purified by cation-exchange and ammonium hydroxide elution were analyzed by C-18 reverse-phase HPLC of the phenylthiocarbamyl derivatized amino acids (Bidlingmeyer et al., 1984). Two independent analyses were performed. One analysis used an automated Applied Biosystems model 130 HPLC system, with an Applied Biosystems PTC column (5 μm C18, 220 × 2.1 mm). Pierce Standard H and Sigma nonprotein amino acid standards were used for analyses done on sweet corn, squash, and sunflower pollens; 250 pmol norleucine was used as an internal standard. Resolution was achieved with a gradient of 120 mM sodium acetate, pH 6.45, to 31.5 mM sodium acetate in 60% acetonitrile at 300 μl/min and 36°C.

A second analysis was performed with dual Waters Associates chromatography pumps, Waters model 660 isocratic gradient solvent programmer, a Nova-

pak C-18 reverse phase column (4 μm, 3.9 mm × 150 mm, 60 Å), Waters model U6K manual injector with 250 μl sample loop, and a Shimadzu CR501 chromatopac integrator. PITC derivatization was performed manually on mixtures of Sigma protein and nonprotein amino acid standards (99+%) using reagent solutions outlined above for PTH derivatization. In a 1.5-ml Eppendorf tube, 10 μl of amino acid standard (5–10 nmol) or pollen extract and 1 μl norleucine internal standard (3–5 nmol) were evaporated to dryness on a Savant SC110 Speed Vac, then 10 μl of TEA solution were added, immediately dried and followed by addition of 20 μl PITC solution, which sat 20 min at room temperature before evaporation. The TEA solution was added and evaporated two more times, and then derivatized samples were stored at −20°C until use. Samples were reconstituted in 100 μl of 6% aqueous acetonitrile for manual injection of 10 μl onto the column. Sweet corn, squash, sunflower, and goldenrod pollen samples were diluted as necessary. Two eluents were used: (A) 140 mM sodium acetate, pH 6.45, with 0.5 ml TEA/liter, titrated to pH 6.3 with glacial acetic acid; and (B) 60% acetonitrile. A linear gradient was used, going from 2 to 53% of eluent B in 40 min at 1.0 ml/min. After each run, the column was flushed with 100% eluent B for 10 min and reequilibrated to initial conditions for 10 min.

Pollen Consumption Bioassay. In a no-choice bioassay, pollen (10 mg) was placed in 11-mm-diameter aluminum boats with four treatments: sweet corn, squash, sunflower, and goldenrod. The pollen was presented to one starved (24 hr) adult female WCR on moistened filter paper in an 11-cm-diameter Petri dish arena. Replicate dishes (N = 15) were placed in an environmental growth chamber for 24 hr at 25°C with a 16L : 8D cycle. Remaining pollen was weighed after drying the boats for at least 1 hr on a hot plate. The fresh weight of pollen consumed was calculated after correcting for water evaporation in control dishes without beetles (N = 5). Separate bioassays were performed with field collected beetles or the nondiapausing strain. A one-way ANOVA was used to test for treatment effects, and the Fisher's Least Significant Difference test was used for pairwise comparison of treatment means. A square root transformation was performed for field WCR to correct for nonnormality of error terms.

Choice Feeding Bioassay. In the choice bioassay, 4 μl of extract or carrier solvent were applied with a Hamilton syringe to 6-mm-diameter disks (27 mm^2) cut from regenerated cellulose membrane filters (pore size 0.45 μm, Schleicher and Schuell) with a standard paper punch. Air-dried disks were stuck on minuten pins above moistened filter paper in a 9-cm-diameter Petri dish with a paraffin wax bottom. Four randomly selected adult WCR, without sex discrimination, were placed in the Petri dish and allowed to feed for 24 hr, with 6 replicates per treatment. Petri dishes were enclosed in plastic boxes and placed in an EGC growth chamber (25°C, 16L : 8D). After 24 hr, the insects were removed, cellulose disks were left to dry on filter paper, and surface areas were measured to the nearest 0.01 mm^2 on an Optomax V image analyzer (Analytical Measuring Systems, Ltd.).

Extract bioassays consisted of three treatments: petroleum ether, methanol, and water extract fractions. Extracts were resuspended in 1.0 ml of solvent, with 4 μl of treatment volume equivalent to 6 mg pollen. For cation-exchange fractions, four treatments were used: 50% aqueous methanol, unbound Dowex 50 water fraction, Dowex 50 ammonium hydroxide eluate fraction, and combined Dowex 50 fractions (1 : 1). Treatment extracts were resuspended in 1.0 ml of solvent and adjusted to pH 6.5–7.0 with HCl, with the 4-μl treatment volume equivalent to 3.6 mg pollen. Treated disks were compared with a respective solvent control disk, with two of each disk per Petri dish. For each treatment, a consumption index (CI) was calculated where CI equals the surface area of solvent control disk (C) minus the surface area of treated disk (T) divided by (C), and represents the percentage of treated disk area consumed relative to the solvent control $[CI = (C - T)/C \times 100]$. A one-way ANOVA was used to test for treatment effects; multiple pairwise comparison of treatment means was done using the Tukey-Kramer Honestly Significant Difference test.

Carbohydrate phagostimulation was assayed using three doses per compound, comparing treated disks with solvent treated controls. Sucrose treatments included doses of 1052, 131, and 26 nmol/disk, while fructose was bioassayed at 888, 444, and 111 nmol/disk, each with five replicates per treatment. A two-way ANOVA was used to test for treatment effects between surface areas of control and treated disks of varying dosage.

No Choice Feeding Bioassays. In a no-choice bioassay (Hollister and Mullin, 1998), 4 μl of treatment solution were applied to a single disk, handled as above, and exposed to two adult WCR. To obtain dose–response relationships for amino acid mixtures, increasing doses of proline–alanine–γ-aminobutyric acid (GABA) (72 : 18 : 10 molar ratio) or β-alanine–asparagine–alanine (47 : 34 : 19) were used. Ratios of amino acids in the mixture were based upon levels from HPLC analysis of sweet corn and squash pollen, respectively. Bioassays consisted of five doses ranging from 10.4 to 853 nmol/disk for the sweet corn mixture and 8 to 610 nmol/disk for the squash mixture. Treatments were replicated 20 times. Disk consumption after 24 hr was measured as before by using the image analyzer, and a CI was calculated based on area of treated disk consumed relative to a standard control disk. Dose–response relationships were analyzed with a simple linear regression model by using standard least squares estimates and log transformed doses. Analyses were done on the linear response range of the log dose response curves (JMP 3.1, SAS Institute), which included three doses for both mixtures. A half-maximal response (ED_{50}), relative to the maximal phagostimulatory response for each mixture, was calculated from the linear regression equation.

To compare phagostimulatory responses between cation-exchange amino acid fractions and pure three component amino acid mixtures, a choice test was used as above. Six treatments were assayed including Dowex 50 ammo-

nium hydroxide eluate fractions from sweet corn, squash, sunflower, and goldenrod extracts (pollen extract treatments represent 3.6 mg pollen equivalents), the proline–alanine–GABA mix at 284 nmol/disk, and the β-alanine–asparagine–alanine mixture at 204 nmol/disk. Treatments were replicated 16 times. A one-way ANOVA was used to test for treatment effects.

RESULTS

Extract Analysis

TLC of methanolic sweet corn, squash, sunflower, and goldenrod pollen extracts indicated, based on spot diameters, the presence of large quantities of free amino acids and simple carbohydrates. Predominant free amino acids by ninhydrin reactivity and cochromatography were typically serine (R_f = 0.21), alanine (R_f = 0.24), GABA (R_f = 0.38), and large quantities of proline (R_f = 0.30), which develops as a yellow spot. For squash pollen, β-alanine and smaller quantities of leucine (R_f = 0.53), phenylalanine (R_f = 0.55), and histidine (R_f = 0.03) were also evident. In corn pollen, the presence of aspartic acid was suggested by cochromatography (R_f = 0.39) and a characteristic bluish ninhydrin response; smaller quantities of valine (R_f = 0.62) were also observed. Suprisingly, in sunflower, histidine, proline, alanine, and aspartic acid predominated without substantial amounts of serine and GABA. For goldenrod, large quantities of histidine and phenylalanine were also indicated by TLC. Water extracts of the four pollens, subsequent to alcoholic extract, contained similar amino acids but at lower concentrations.

TLC of PTH-derivatized amino acids with solvent system 2 independently confirmed the predominance of proline (R_f = 0.61), serine (R_f = 0.12), alanine (R_f = 0.31), GABA (R_f = 0.40), and β-alanine (R_f = 0.49) in methanol extracts and ion-exchange ammonium hydroxide extract fractions from sweet corn, squash, and sunflower pollens. For corn pollen, cochromatography additionally indicated the presence of large quantities of asparagine (R_f = 0.06) as well as the monoamine, ethanolamine (R_f = 0.22). Dominance profiles for PTH-derivatized amino acids followed the same pattern as for free amino acids. This technique was particularly useful with solvent system 1 in distinguishing GABA (R_f = 0.62, 0.29) from its α-aminobutyric acid (R_f = 0.60, 0.24), β-aminobutyric acid (R_f = 0.67, 0.34), and α-aminoisobutyric acid (R_f = 0.59) isomers. Aminobutyric acid isomers give both a PTH and an acyclic product, except for the isobutyric acid isomer, which gives a single cyclic PTH derivative.

HPLC analyses of cation-exchange ammonium hydroxide eluates for all four pollen species are represented in Table 1. These analyses quantitatively confirm the free amino acid profiles derived from TLC analyses, verifying the consistent predominance of proline, serine, alanine, β-alanine, and GABA. The profiles of amino acids differ, however, across the four pollen species.

TABLE 1. HOST POLLEN FREE AMINO ACID CONCENTRATIONS BASED ON HPLC ANALYSIS OF AQUEOUS METHANOL ION-EXCHANGE FRACTIONS

Amino acid	Amino acid (μmol/g pollen)[a]			
	Sweet corn	Squash	Sunflower	Goldenrod
Proline	66.22 ± 15.92	11.72 ± 1.91	19.12 ± 2.08	25.66 ± 7.70
Alanine	5.01 ± 1.32	13.39 ± 2.19	6.25 ± 0.63	2.64 ± 0.23
Serine	7.78 ± 1.28	3.90 ± 0.24	0.25 ± 0.02	4.66 ± 0.95
GABA	4.26 ± 0.46	7.98 ± 1.14	0.28 ± 0.02	0.82 ± 0.12
Asparagine	3.03 ± 0.70	22.63 ± 2.89	7.16 ± 1.05	[b]
β-Alanine	0.74 ± 0.68	33.24 ± 1.19	0.54 ± 0.06	[b]
Histidine	0.26	1.25 ± 0.06	34.96 ± 4.49	3.73 ± 0.58
trans-Hydroxyproline	0.28 ± 0.01	[b]	1.19 ± 0.08	5.60 ± 1.84
Aspartic acid	1.60 ± 0.20	4.17 ± 1.15	7.39 ± 1.11	0.23 ± 0.08
Glutamic acid	0.98 ± 0.79	0.80 ± 0.28	1.92 ± 0.74	0.69 ± 0.39
Glycine	1.39 ± 0.26	2.95 ± 0.48	0.64 ± 0.10	0.71 ± 0.06
Arginine	0.31 ± 0.19	0.67 ± 0.12	0.20 ± 0.14	0.30
Threonine	0.78 ± 0.58	[b]	0.11	[b]
Valine	0.94 ± 0.27	2.64 ± 0.18	0.59 ± 0.08	0.35 ± 0.15
Methionine	0.29 ± 0.09	0.26 ± 0.15	0.07	[b]
Phenylalanine	0.25 ± 0.06	1.60 ± 0.38	0.10	3.60 ± 1.91
Leucine	0.91 ± 0.18	1.85 ± 0.41	0.22 ± 0.02	0.17 ± 0.06
Lysine	0.50 ± 0.17	1.96 ± 0.33	2.60 ± 0.17	1.21 ± 0.27
Tyrosine	0.66 ± 0.25	1.60 ± 0.66	0.47 ± 0.33	0.36 ± 0.08
Total	96.17	112.61	84.06	50.73

[a]Quantities represent extrapolation to fresh pollen content from HPLC analyses. Sweet corn, squash, and sunflower values are the average of three separate analyses ± SE. Goldenrod values are the average of two separate analyses ± SE.
[b]Compounds absent, or present below detectable levels.

Large concentrations of simple carbohydrates were present in pollen methanol extracts. Cochromatography indicated the presence of glucose and sucrose in sweet corn, squash, and sunflower pollen. Stachyose was present in much smaller concentrations in sweet corn pollens. Cation-exchange chromatography of aqueous methanol extracts resulted in complete separation of the amino acids and carbohydrates. The ammonium hydroxide eluate and unbound water-wash fractions were shown by TLC to contain predominantly amino acids and mono- and disaccharides, respectively.

The quaternary ammonium compound glycine betaine was evident among NH_4OH-displaceable cations from Dowex 50 bound components of the sunflower pollen aqueous methanol extract, based on cochromatography at R_f = 0.17 and its reactivity with Dragendorf's reagent and iodine (Storey and Wyn Jones, 1977). The carotenoid, β-carotene, was indicated from the sunflower

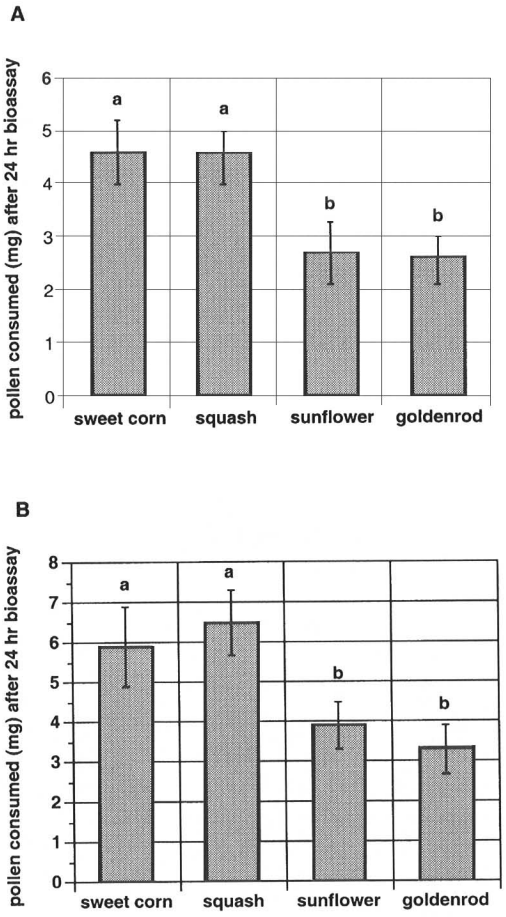

FIG. 1. Amount of pollen consumed by western corn rootworm in a 24-hr bioassay: (A) field-collected western corn rootworm adults; (B) nondiapausing western corn rootworm laboratory strain. Different letters above the bars ± SE indicate significant differences between treatment means at $\alpha = 0.05$ for field WCR, and $\alpha = 0.07$ for nondiapausing WCR.

pollen petroleum ether extract based upon three absorbance maxima peaks at 424, 444, and 472 nm (Harborne, 1984).

Feeding Bioassay

Pollen Consumption. Adult WCR readily fed on all four pollen species (Figure 1). For field-collected beetles (Prob < F, 0.0028), consumption of sweet

TABLE 2. PHAGOSTIMULATORY RESPONSE OF ADULT WESTERN CORN ROOTWORM TO HOST POLLEN SOLVENT EXTRACTS

Pollen	Consumption index of extract[a]		
	petroleum ether	methanol	water
sweet corn	36.8 ± 5.3[a]	74.8 ± 7.3[b]	60.9 ± 3.4[b]
squash	36.0 ± 7.6[a]	82.4 ± 2.8[b]	61.8 ± 5.8[b]
sunflower	34.9 ± 9.6[a]	96.5 ± 1.9[b]	85.9 ± 4.6[b]
goldenrod	19.6 ± 3.8[a]	76.1 ± 2.9[b]	71.2 ± 3.2[b]

[a]Consumption index values represent the percentage of treated disk area consumed relative to the solvent control ± SE. Significant differences ($P < 0.05$) between treatment means ($N = 6$) within a pollen species indicated by different letters.

corn and squash pollen were significantly greater than for sunflower and goldenrod pollen at $\alpha = 0.05$ level. Nondiapausing beetles (Prob < F, 0.0198) followed the same pattern.

Extract Phagostimulation. For all pollen species, there was significantly greater WCR consumption on methanol- and water extract-treated disks than on the petroleum ether treatment (Table 2). Methanol solvent extracts appeared to stimulate higher consumption than water, but the difference was not significant.

Ion-Exchange Fractions. For all pollen species, the pattern of stimulation for aqueous methanol cation-exchange fractions was the same (Table 3). For sweet corn, squash, and goldenrod pollen extracts, the original aqueous methanol, Dowex 50 ammonium hydroxide, and combined Dowex 50 extracts stimulated higher consumption than the Dowex 50 water fraction. For sunflower

TABLE 3. PHAGOSTIMULATORY RESPONSES OF ADULT WESTERN CORN ROOTWORM TO CATION-EXCHANGE FRACTIONS FROM AQUEOUS METHANOL EXTRACTS OF POLLENS

Pollen	Consumption index of ion-exchange fraction[a]			
	50% aqueous methanol	Dowex 50 ammonium hydroxide	Dowex 50 water	Dowex 50 combined fractions
Sweet corn	65.0 ± 9.2a	74.7 ± 9.1a	8.4 ± 4.6b	80.2 ± 6.1a
Squash	76.7 ± 6.3a	69.1 ± 6.4a	28.5 ± 9.2b	66.4 ± 4.1a
Sunflower	87.1 ± 3.4a	75.9 ± 11.8ab	37.9 ± 11.7b	67.6 ± 10.4ab
Goldenrod	90.5 ± 3.3a	87.2 ± 4.4a	4.2 ± 2.0b	91.7 ± 2.0a

[a]Consumption Index values represent the percentage of treated disk area consumed relative to the solvent control ± SE. Significant differences ($P < 0.05$) between treatment means ($N = 6$) within a pollen species represented by different letters.

pollen, the pattern of stimulation was the same, but the difference was significant only for the original aqueous methanol and Dowex 50 water fractions.

Carbohydrate Phagostimulation. For fructose, there were no significant main effects for either dose (Prob > F, 0.571) or treatment (Prob > F, 0.192); calculated consumption indices were 0.4, 5.0, and 7.8% for the low, medium, and high doses, respectively. For sucrose, there was no significant main effect for dose (Prob > F, 0.970), and the main effect for treatment was marginally not significant (Prob > F, 0.0566); calculated CI for the low, medium, and high sucrose doses were 1.8, 1.8, and 3.4%, respectively.

Amino Acid Mix Dose–Response

Both ternary mixtures of dominant free amino acids representative of sweet corn and squash pollen profiles stimulated strong feeding activity with a clear dose-dependent response (Figure 2). The maximum response was considered as the mean response from the two highest doses in the saturation portion of the dose–response. The linear response range included doses up to 284 nmol total amino acid/disk, with a threshold, the x intercept of the linear portion of the dose–response curve, of 24 nmol/disk for the sweet corn mixture. For the squash mixture, the linear response range included doses up to 204 nmol total amino acid/disk, with a threshold of 17 nmol/disk. Half maximal (ED_{50}) values were 73.84 and 57.90 nmol total amino acid/disk for the sweet corn and squash mixtures, respectively. Response slopes for both mixtures were similar (Table 4).

Comparison of Cation Exchange Fractions and Amino Acid Mixtures. All six treatments gave strong phagostimulatory activity (Figure 3). There was no significant effect of treatment (Prob > F, 0.0618).

DISCUSSION

Utilization of pollen as a food source occurs widely in insects. Pollen is a highly nutritious resource (Stanley and Linskens, 1974) and has a significant effect on reproduction in many insects (Gilbert, 1972; Kirk, 1985; Pesho and Van Houten, 1982). For *Diabrotica*, egg production is greater on diets including corn pollen than on immature tassels or senescing silks (Elliott et al., 1990; Naranjo and Sawyer, 1987). Despite these and general observations on the pollen feeding behavior of *Diabrotica*, little is known of this interaction. Here, we present the first quantitative investigation of *Diabrotica* pollen consumption behavior. Greater feeding by western corn rootworm on sweet corn and squash pollen relative to the Asteraceae species suggested preferences among host pollens normally available in the field, although a no-choice experimental design was used. This observed difference in pollen feeding led to our investigation of chemical cues driving pollen consumption.

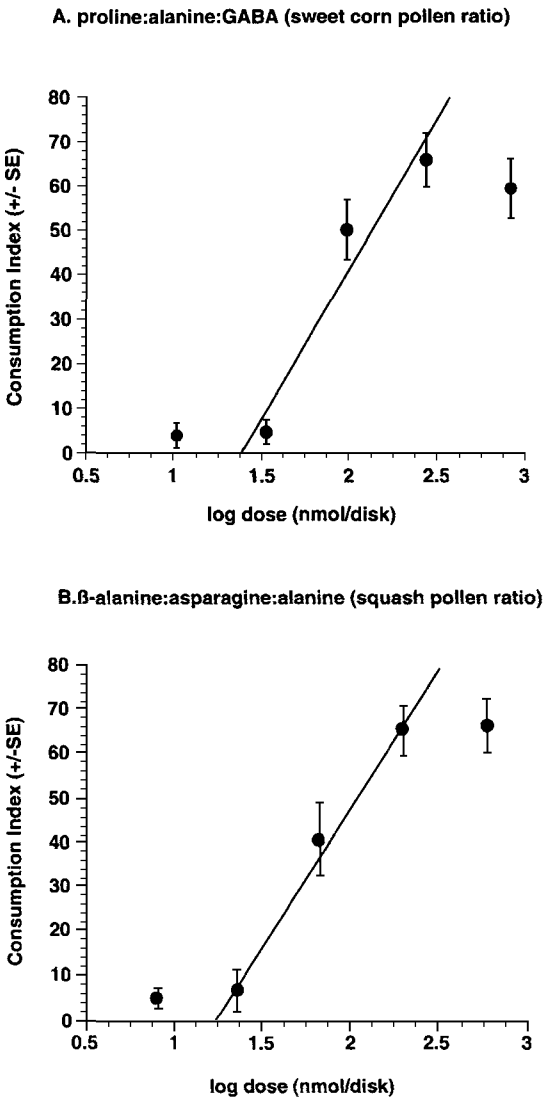

FIG. 2. Adult WCR dose–consumption relationships for cellulose disks treated with ternary mixtures representing dominant amino acids in two host pollen species. (A) Log dose–response curve for a mixture of proline, alanine, and GABA in ratios derived from HPLC analysis of sweet corn pollen extracts. A regression line is fitted to the linear range of the dose-response. (B) Log dose–response curve for a mixture of β-alanine, asparagine, and alanine in ratios derived from HPLC analysis of squash pollen extracts. $N = 20$/dose.

TABLE 4. PHAGOSTIMULATORY DOSE–RESPONSE RELATIONSHIPS FOR POLLEN AMINO ACID MIXTURES ON ADULT WESTERN CORN ROOTWORM

Amino acid mixture[a]	R^2	ED_{50} (nmol/disk)[b]			Maximal response[c]	Slope of response[d]
		Lower limit	Estimate	Upper limit		
Proline–alanine–GABA	0.48	52.3	73.8	96.8	65.6 ± 5.6	17.5
β-Alanine–asparagine–Alanine	0.55	45.6	59.2	75.1	65.9 ± 6.1	17.4

[a]Ratios of amino acids within mixtures based on levels found in host pollen from HPLC analyses.
[b]Lower and upper limits are based upon 95% confidence interval predictions.
[c]Mean maximum disk area consumed (consumption index ± SE) calculated from the highest stimulatory doses within each mixture.
[d]Slope of response (mm^2 of disk consumed) on log dose (nmol/disk) from linear regression analysis.

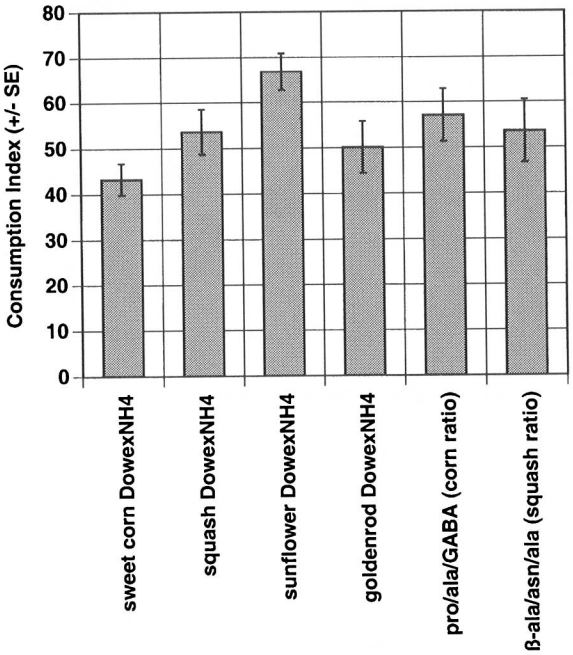

FIG. 3. Consumption of host pollen cation-exchange fractions of combined methanol–water extracts and of amino acid mixtures approximating dominant amino acids from sweet corn and squash pollens (mean ± SE, N = 16/treatment).

Amino acids (Stanley and Linskens, 1974; Carisey and Bauce, 1997) and carbohydrates (Stanley and Linskens, 1974; Pacini, 1996) have consistently been identified as common pollen constituents. Variation in amino acid profiles across pollen species has been reported previously (Bathurst, 1954; Bieberdorf et al., 1961; Stanley and Linskens, 1974). These studies indicate proline is one of the most abundant free amino acids found in pollen, and our analyses confirm this. These primary metabolites, along with nucleotides and lipids (Stanley and Linskens, 1974) have all been identified as phagostimulants for various insects (Bernays and Chapman, 1994). Secondary chemicals such as flavonoid pigments (Stanley and Linskens, 1974), phenolic compounds (Meurer et al., 1986; Niester-Nyveld et al., 1997), and terpenoids (Stanley and Linskens, 1974; Carisey and Bauce, 1977) are present in pollen. Only rarely have substances toxic to bees and other pollen-feeding insects, such as saponins and alkaloids, been found (Stanley and Linskens, 1974; Detzel and Wink, 1993). The large amounts of amino acids, carbohydrates, and minerals relative to secondary substances give pollen a high nutritive value for pollinivorous insects (Carisey and Bauce, 1997).

Our sequential surface extraction of pollen with solvents of increasing polarity isolated alcohol and water extracts with high phagostimulatory activities. This is consistent with previous work that localized insect pollen feeding or oviposition bioactivity to crude aqueous or alcohol extracts (Delisle et al., 1989; Jayanth et al., 1993; Wacht et al., 1996). Further fractionation by cation-exchange and silica gel chromatographies led to identification of amino acids as the dominant phagostimulants for WCR from host pollens.

Sugars, particularly sucrose and fructose, are generally considered to be the most effective nutrient phagostimulants. The phagostimulatory response to amino acids has long been known (Thorsteinson, 1960; Hsiao and Fraenkel, 1968), but is considered to be low compared to sugars, resulting in the generalization that, for most insects feeding on plants, phagostimulatory effects are likely to be dominated by sugars (Bernays and Chapman, 1994). For adult WCR, this generalization does not hold true. Cation-exchange extract fractions containing bulk mono- and disaccharides resulted in less phagostimulatory activity than extract fractions containing bulk free amino acids, and we found no significant effect of carbohydrate treatment on consumption in cellulose disk bioassays.

Since the study by Fraenkel (1959) and subsequently that of Ehrlich and Raven (1964), most attention has been given to secondary metabolites as attractants, feeding deterrents, or toxicants driving insect-host plant associations. A subsequent debate on the evolution of host associations has involved whether secondary chemicals act at the peripheral or central nervous system affecting insect behavior (Bush, 1975; Futuyma, 1983; Jermy, 1993) versus a postingestive toxic or digestibility-reducing effect (Ehrlich and Raven, 1964; Feeny, 1976; Berenbaum, 1986; Bernays and Chapman, 1994) in determining patterns of host use. Berenbaum (1995) argues for more attention given to the role of primary

metabolites in insect–plant interactions, with a focus on the resistance against herbivores that primary metabolites can confer, particularly in the form of low nutrient quality. She does not address chemosensory and behavioral responses to primary metabolites. In the adult *Diabrotica*–pollen interaction, it is apparent that amino acid primary metabolites are a major phagostimulatory factor, likely having an effect at the peripheral sensory or central nervous system level to influence feeding behavior (Mullin et al., 1994).

We demonstrate here a strong association of WCR phagostimulation by pollen extracts with free amino acid chemistry. With our sequential extraction, the lipoidal components of the "pollenkitt" covering the exine, simple sugars, and free amino acids are removed by petroleum ether, methanolic, and water extraction, respectively. Carbohydrates and amino acids, which are internal components of the pollen grain (Dobson, 1988; Pacini, 1996; Sperenza et al., 1997), are likely extracted through exine pores. Our observations of pollen feeding by adult *Diabrotica* indicate that after a 24-hr period, uneaten pollen is often present in a slurry of liquid due to beetle salivation. It is probable that beetles feeding on a pile of pollen are stimulated to feed by extracted pollen chemicals, particularly low-molecular-weight primary metabolites.

Soldaat et al. (1996) have suggested that primary metabolites have a role in the host specificity of *Tyria jacobae*, based upon observations on distinct proportions of cuticular amino acids for four species of *Senecio*, two hosts and two nonhosts. We have similarly proposed that *Diabrotica*–pollen host associations can be attributed to stimulatory responses to pollen chemicals, particularly amino acids (Hollister and Mullin, 1998), based upon behavioral and electrophysiological responses to predominant host pollen amino acids. Here, we demonstrate strong phagostimulatory responses to low-molecular-weight, neutral amino acid mixtures approximating distinct proportions found in a number of host pollens based upon HPLC analysis. This study provides further evidence that amino acids, and particularly mixture interactions, are largely responsible for the peripheral sensory code that drives *Diabrotica*–pollen feeding behavior.

The peripheral sensory code and the central processing of that code, which drives feeding behavior in insects, has long been a topic of debate (Dethier and Crnjar, 1982; Schoonhoven, 1987). Our observation that simple mixtures of amino acids, approximating ratios found in host pollens, stimulates equally strong phagostimulation relative to crude polar extract fractions prompts investigation into the sensory code driving pollen-feeding behavior in this insect. We have shown that individual amino acids stimulate dose-dependent responses in *Diabrotica* galeal chemosensilla that directly correlate with respective consumption behavior (Hollister and Mullin, 1998). Investigations into chemosensory responses to mixtures of amino acids will provide insight into a functional sensory code for these beetles. Lower level phagostimulation in petroleum ether

extracts suggests that other phagostimulatory pollen chemicals may interact with small, neutral amino acids to drive pollen feeding behavior in adult *Diabrotica*.

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EFFECTS OF PERUVIAN MAIZE EXTRACTS ON GROWTH, DEVELOPMENT, AND FECUNDITY OF THE EUROPEAN CORN BORER

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Abstract—Twelve Peruvian maize, *Zea mays*, accessions were selected because of their relatively high level of field resistance to first-generation European corn borer (ECB), *Ostrinia nubilalis*, larval leaf-feeding. Water extracts of freeze-dried, powdered, leaf tissue were incorporated into a standard ECB diet, fed to larvae, and the effects on larval growth, development, and fecundity were measured. Larval and pupal weights were monitored as were the time elapsed in the larval, pupal, and adult stages. Adult fecundity and egg fertility were recorded. The experiment was a randomized block design (larvae and pupae) or a completely randomized design (adults) and analyzed with ANOVA ($\alpha = 0.05$). Pairwise comparisons were made between groups of insects grown on diets containing extracts from the Peruvian lines, a standard diet, or diets containing extracts of a known susceptible inbred, and a known resistant inbred line. Survival was analyzed with a chi-squared test ($\alpha = 0.05$). Two Peruvian accessions significantly reduced female larval and pupal weights, extended pupal and adult development time, and decreased survival of pupae and adults. Water extracts also had a pronounced impact on males; two accessions significantly reduced pupal weight and extended the time required to pupate, and one

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reduced male survival to adults. The results indicate that water-soluble factors from resistant Peruvian accessions inhibit the growth, developmental time, and survival of ECB. These resistance factors could be useful in the development of maize germplasm with insect-resistant traits.

Key Words—European corn borer, *Ostrinia nubilalis* (Hübner), growth, development, fecundity, oviposition, behavior, eggs, DIMBOA, water extract, plant extract, maize, Peruvian maize, maize accessions, host plant resistance.

INTRODUCTION

The European corn borer, *Ostrinia nubilalis* (Hübner) (ECB) is a significant pest of maize, *Zea mays* L., and other crops in the United States (Mason et al., 1996). One approach to reduce yield losses caused by ECB is to develop inbred lines of maize with natural physical and/or chemical resistance that can be used as parents of hybrids utilized by farmers (Guthrie and Barry, 1989). New sources of maize resistance to ECB have been identified recently by field evaluating Peruvian maize from the extensive collections of germplasm at the United States Department of Agriculture North Central Regional Plant Introduction Station (NCRPIS) (Abel et al., 1995). These exotic accessions have proven to be a rich resource for germplasm with new types of resistance against ECB and other maize pests (Davis et al., 1988; Wilson et al., 1995).

Many resistance mechanisms in plants result from the production and accumulation of chemicals that repel or deter insect herbivores (nonpreference), or these chemicals may inhibit growth and development (antibiosis) of those insects that feed on the plant tissue (Maxwell and Jennings, 1980; Panda and Khush, 1995). A well-studied example is the protection some maize genotypes have against ECB larvae because they synthesize hydroxamic acids such as DIMBOA [2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one] and related compounds (Klun et al., 1970). Leaf concentrations of DIMBOA decrease as the plants develop in all DIMBOA-related maize genotypes studied thus far, so this resistance mechanism fails to protect plants from ECB attack throughout the growing season in the United States. New genotypes are continually being created by plant breeders and entomologists to improve yields, generate high-quality agronomic traits, and maintain resistance to pests over the entire field season. Newly discovered maize germplasm with resistance to ECB and other pests may have novel, as yet undiscovered, biochemical pathways that could serve a significant role in the plant's protection.

Peruvian maize collections maintained at the NCRPIS extend genetic diversity beyond the uniformity of lines adapted solely to North America and thus provide reserves of seed with insect-resistant traits. More than 1600 Peruvian genotypes were evaluated for first-generation ECB resistance, and 11 entries showed

promise as a new genetic reservoir of insect resistance (Abel et al., 1995; Wilson et al., 1995). Chemical evaluation of the Peruvian maize leaf tissue revealed that the amounts of DIMBOA present were not high enough to account for the ECB leaf-feeding resistance. Identification of a new chemical resistance mechanism would be an important step toward developing maize germplasm that is resistant to ECB feeding. The objective of the present study was to evaluate whether ECB resistance in the Peruvian accessions observed by Abel et al. (1995) is caused by chemical factors and to determine the genotypes that contain defensive chemicals at sufficient levels to cause inhibition of growth, development, and fecundity in ECB.

METHODS AND MATERIALS

Extraction of Plant Material. Eleven Peruvian lines of maize (Ames 10623, PI 503720, PI 503722, PI 503723, PI 503725, PI 503727, PI 503728, PI 503731, PI 503764, PI 503806, and PI 503849), which were field tested and found resistant to ECB larval leaf-feeding by Abel et al. (1995), were selected for study along with a resistant line PI 485320 (Abel and Wilson, unpublished observations), a susceptible check (WF9), and a resistant check (CI31A) (Table 1). All accessions were planted at the Plant Introduction Station at Ames, Iowa, on May 15, 1995. Leaves for chemical extraction were prepared following the method of Wilson and Wissink (1986). When the maize reached the V7–V8 leaf stage (Ritchie et al., 1986) (June 28, 1995), ca. 40 plants of each accession were cut off ca. 15 cm below the whorl, packaged by accession, and frozen immediately at −20°C. Leaves were removed from refrigeration and freeze dried in a LABCONCO model 5 freeze dryer (Kansas City, Missouri), milled to a fine powder by using a Thomas Wiley Laboratory Mill, model No. 4 (Philadelphia, Pennsylvania), and stored again at −20°C in 0.5-liter glass jars until extraction. Later, powdered leaf material of each accession (20.25 g) was extracted with 500 ml of distilled water in a commercial Waring Blender (model 36BL22, New Hartford, Connecticut); after filtration (Whatman No. 1, Whatman International, Maidstone, Kent, England) the liquid extracts were frozen at −20°C and lyophilized to reduce the volume in a LABCONCO FreeZone R 18 Liter Freeze Dry System model 77550-10. The remaining residues were stored at −20°C until use in the growth, development, and fecundity studies (Table 1).

Insects. Laboratory-reared ECB larvae that were ca. eight generations removed from the field and reared according to the methods of Reed et al. (1972) were used in this study. Adults eclosed in specially designed cages (58.7 × 58.7 × 63.7 cm) made of angle-and-strap aluminum frame (1.9 and 2.3 cm, respectively) covered on the sides and bottom with 16 × 18 mesh brass cloth (Binder and Robbins, 1996). The top of the cage was covered with 5 × 5 mesh galva-

TABLE 1. ECB LARVAL RESISTANCE RATINGS FOR PERUVIAN MAIZE ACCESSIONS AND AMOUNT OF WATER-EXTRACTABLE RESIDUE INCORPORATED INTO 200 GRAMS OF ARTIFICIAL DIET

Accession	Mean leaf-feeding ratings[a]	Extract weight (g)[b]	Peruvian maize race
WF9	7.1 a	3.0	Susceptible check
PI 503725	3.2 de	3.4	Mochero
PI 503806	3.2 de	3.0	Alazan
PI 503849	3.2 de	3.2	Alazan
PI 503764	3.0 def	3.6	Mochero
Ames-10623	3.0 def	3.9	Arizona
PI 503720	2.9 def	3.3	Mochero
PI 503722	2.9 def	3.7	Mochero
PI 503728	2.9 def	3.7	Mochero
PI 503727	2.8 ef	3.2	Mochero
PI 503723	2.6 efg	2.6	Mochero
PI 503731	2.2 fg	3.2	Mochero
CI31A	1.8 g	3.0	Resistant check
PI 485320	4.0	3.3	Mochero

[a]Means followed by the same letter are not significantly different according to the LSD test ($P = 0.05$) ($N = 8$). Visual ECB leaf feeding ratings on manually infested plants (1–9 scale; Guthrie et al., 1960). These ratings were taken by Abel et al. (1995) during the 1992 growing season at the USDA Plant Introduction Station at Ames, Iowa (except PI 485320, which was completed on a later date and therefore not included in the analysis).
[b]Extracted from 20.25 g of powdered dried leaf.

nized hardware cloth, which permitted oviposition on the underside of wax paper sheets positioned on top of the cage. Two feeding stations were included in each cage. One was a cotton pad suspended from a brass rod 19.5 cm from the top of the cage and moistened daily with water. The other, a molded plastic unit (10.3 cm^2) with 16 wells (each with a capacity of 1 ml), was located on the bottom of the cage and its wells were filled with 1.4% (w/v) agar gel containing 39.4% (w/v) sucrose (Leahy and Andow, 1994). Egg masses were removed from the waxed sheets and placed in 70-ml screw-capped glass bottles. The bottles were incubated at 27°C, 75–80% relative humidity, and with continuous light until larvae hatched. ECB larval neonates within 12 hr of hatch were used in all tests.

Diet Formulation with Maize Extracts. Standard ECB diet was prepared according to Reed et al. (1972). Each Peruvian maize, WF9, and CI31A dry extract was dissolved in 20 ml distilled water and mixed into a 200-ml aliquot hot liquid ECB diet (50–56°C). Control diet consisted of 200 ml of standard ECB diet plus 20 ml of distilled water. Each diet was poured into Petri dishes to cool and harden overnight at room temperature. A total of 15 diets were prepared and stored for ca. seven nights at 4.4°C until the experiment was started. Diets were warmed to room temperature before use.

ECB Larval Growth and Development Studies. Three neonate larvae were transferred to each six-dram vial with experimental diet plugs (1.5 cm diameter × 1.2 cm deep, 4 g), and the vial was tightly plugged with a piece of autoclaved cotton (nonabsorbent, nonsterile cotton, Acco, Valley Park, Missouri). Vials were incubated at 27° ± 2°C, relative humidity of 75 ± 5%, with continuous light, in a Percival Incubator (model CE-2, Boone, Iowa). After six days, the two smaller larvae were removed, leaving the most robust individual in each vial. Larval weights were measured after 13 days (±0.1 mg) on a Mettler AE 100 Electronic Balance (Hightown, New Jersey). The balance interfaced with an IBM PS 2 computer (International Business Machines Corp., Armonk, New York) to record larval weight automatically in QuickBasic 1.1 (Microsoft Corp., Redmond, Washington). Data were transferred to Microsoft Excel 5.0 for tabulation. On the day following pupation, each ECB was removed from the vial, weighted (±0.1 mg) on an Ohaus GA 200 D balance (Florham Park, New Jersey), and transferred to a 33-cc plastic cup (covered with a plastic snap-on lid) to monitor for adult eclosion.

ECB Oviposition Studies. Each adult female was paired with a male from the general colony in a 14 × 18 mesh brass cloth cylindrical mating cage, ca. 8 cm height × 8 cm diameter, with a brass cloth bottom and a 4 mesh galvanized hardware top (Kira et al., 1969). The top was covered with a glass petri dish, which served as an oviposition substrate. Cages were kept in an incubation chamber at 16L : 8D; 24.5 (day) : 18.5 (night) ± 2.5°C; 85 ± 10% relative humidity. Optimum humidity was maintained by placing the cages on water-soaked cotton (Acco sterile absorbent cotton). Following mating, female ECB moved to the top of the cage and deposited egg masses on the underside of the Petri dish. Each female was considered successfully mated if she produced fertile eggs. Petri dishes were changed each day; the fecundity (number and size of egg masses for each female) was monitored by measuring each egg mass with a digital analyzer (Decagon Industries Monochrome AgVision Imaging System, Pullman, Washington) (Binder and Robbins, 1996). Petri dishes with egg masses were incubated under continuous light at 24.5 ± 1.5°C and 96 ± 2% relative humidity to facilitate optimum larval hatch. Fertility or the percentage of viable eggs (= hatched larvae) was determined by visual examination under a 10× dissecting microscope (model 41, American Optical, Buffalo, New York).

Male adults from the test diets were not used in the mating portion of the experiment because female mating, longevity, and oviposition were the foci of the study.

Statistical Design and Analysis. We used a randomized block design with 15 replicates where an experimental unit consisted of two vials with one larva per vial. Male and female data were analyzed separately. Mortality and differences in sexes in each experimental unit resulted in incomplete data. Treatment means in the randomized block analysis were adjusted for missing data, and

significance tests were adjusted for all pairs of adjusted means. PROC GLM and LSMEANS (SAS Institute, Inc., Cary, North Carolina; www.sas.com) were used for computing the analysis. Differences from expected values for survival to pupae and adults were analyzed with a chi-square test using PROC FREQ (SAS Institute, Inc.). Adult longevity, fecundity, and fertility were analyzed in a completely randomized design with PROC GLM.

RESULTS

Incorporation of water-extractable compounds from dried leaf tissue of some Peruvian accessions and CI31A caused reductions in ECB larval and pupal weights, delays in development, and reduced fecundity as compared with larvae reared on WF9 or the standard diet (Tables 2–4). ANOVA and chi-square evaluations indicated significant differences for all female larval and adult variables except fertility. Males also were affected by Peruvian maize extracts; pupal weight, pupal development time, and survival to adults were affected. The data show that there were no differences between treatments in percentage of larvae of either sex surviving to the pupal stage, and, therefore, Peruvian extracts impaired development of ECB primarily in the pupal and adult stages.

Of the 12 Peruvian lines tested, water extracts of PI 485320 and PI 503723 had the most deleterious effect on female ECB larval weight and development but no effect on male larval weight (Table 2). Female 13-day larval weights for individuals reared on PI 485320 and PI 503723 diets were lower than those reared on the WF9 diet, but their weights were not different from those reared on the resistant check, CI31A. Female larvae that were reared on diet incorporating water extracts of the remaining accessions were not different in weight from those reared on standard diet (Table 2).

Measurement of pupal weight was a reliable indicator of ECB performance because, compared to larval weight, it was less variable. The stability of the pupal weight proved useful to gauge differences in ECB performance. A comparison of pupal weight, therefore, was used to evaluate differences in the effects of extracts of Peruvian accessions. Mean pupal weight of females reared on diets containing water extracts of accessions PI 503723 and PI 503720 were lower than those reared on standard diet or diet containing water extracts of WF9 and PI 503806, but not different from females reared on diet with water extract of CI31A (Table 2). Mean pupal weight of females reared on PI 485320 was the lowest of all treatments tested and even lower than mean pupal weight of females reared on the resistant check CI31A (Table 2). Mean pupal weight of males reared on diets containing water extracts of Peruvian accessions PI 503723 and PI 485320 were significantly lower than those reared on WF9 diet but not significantly different from males reared on CI31A (Table 2).

TABLE 2. LARVAL AND PUPAL WEIGHTS OF ECB REARED ON DIETS CONTAINING WATER EXTRACTS OF PERUVIAN MAIZE ACCESSIONS, INBREDS WF9 AND CI31A, AND STANDARD CONTROL DIET[a]

Treatment	Thirteen-day larval weight (mg) ± SE				Pupal weight (mg) ± SE			
	Male	*N*	Female	*N*	Male	*N*	Female	*N*
PI 503806	82.9 ± 3.6 a	14	120.9 ± 4.9 a	15	69.2 ± 2.9 a	12	96.6 ± 3.6 a	15
WF9 (susceptible check)	85.0 ± 4.0 a	11	115.0 ± 4.8 a	19	72.3 ± 3.3 a	10	95.1 ± 3.5 a	19
PI 503731	85.1 ± 3.9 a	10	113.3 ± 4.6 a	20	67.4 ± 3.2 ab	10	92.3 ± 3.4 a	20
Standard Diet	86.5 ± 3.6 a	12	116.3 ± 4.8 a	15	69.0 ± 2.9 a	12	91.7 ± 3.5 a	15
Ames 10623	83.0 ± 3.9 a	10	114.4 ± 5.2 a	17	73.5 ± 3.2 a	10	91.3 ± 3.8 a	17
PI 503727	86.6 ± 4.0 a	9	111.4 ± 5.1 a	17	70.3 ± 3.2 a	9	90.2 ± 3.7 a	17
PI 503722	85.3 ± 3.8 a	10	113.5 ± 4.4 a	20	66.1 ± 3.1 abc	10	89.8 ± 3.2 a	20
PI 503849	79.3 ± 3.6 a	12	107.9 ± 4.8 ab	16	70.2 ± 2.9 a	12	87.6 ± 3.5 ab	16
PI 503764	78.2 ± 3.3 a	16	106.4 ± 5.1 ab	13	60.2 ± 2.7 abc	16	85.0 ± 3.7 ab	13
PI 503725	82.9 ± 3.7 a	11	111.0 ± 4.7 a	19	65.6 ± 3.0 abc	11	83.2 ± 3.4 ab	19
PI 503728	78.2 ± 3.4 a	13	97.7 ± 4.7 abc	16	63.1 ± 2.8 abc	13	77.7 ± 3.4 abc	16
PI 503720	76.2 ± 4.0 a	10	96.4 ± 4.6 abc	19	61.2 ± 3.2 abc	10	71.3 ± 3.4 bc	19
PI 503723	69.5 ± 4.0 a	9	87.7 ± 4.5 bc	20	50.4 ± 3.3 c	9	68.8 ± 3.3 bc	20
CI31A (resistant check)	74.9 ± 4.1 a	10	90.6 ± 4.6 bc	19	57.7 ± 3.3 abc	10	64.3 ± 3.4 c	19
PI 485320	73.5 ± 4.0 a	10	81.5 ± 4.6 c	19	52.1 ± 3.2 bc	10	54.2 ± 3.4 d	19

[a]Thirteen-day larval and pupal weights are expressed as the means (± SE). Each category was analyzed with ANOVA ($\alpha = 0.05$), and pairwise comparisons within each category were made with an LSMEANS test ($\alpha = 0.05$). Values in each category followed by the same letter indicate means that were not significantly different.

Addition of water extracts of some Peruvian accessions to diets extended larval development time, pupal development time, and the overall time required to reach adult stage (Table 3). Extracts of accessions PI 503723 and PI 485320 extended overall development time for females as compared to females reared on standard diet or on diet with extract from leaves of the susceptible check WF9 (Table 3). Females reared on diet with extracts of either PI 503723 or PI 485320 had overall development times that were not significantly different from females reared on diets with extracts of leaves of the resistant check CI31A (Table 3). Extracts of some Peruvian accessions also shortened adult longevity for females as compared to females reared on standard diet or on diet with extract from leaves of the susceptible check WF9 (Table 4).

Water extracts of Peruvian accessions had a pronounced impact on male development times (Table 3). The extract of PI 503723 and PI 485320 extended male larval development time when compared with males reared on standard diet. There was no difference among diets with extracts of Peruvian or WF9 leaf for male pupal development time or in the overall time for development to the adult stage (Table 3).

ECB survival to the pupal stage, 86.7% or greater on all diets tested, was not affected by the type of accession (Table 4). Survival from the pupal to the adult stage, however, was affected by the type of accession in both males and females (Table 4). When the chi-square test for male survival to adults was analyzed without the PI 485320 group, differences among males reared on the remaining 14 treatments were eliminated. This test thus showed that PI 485320 was responsible for the observed differences in male survival from the pupal to the adult stage. Female survival from pupae to adults, in contrast, separated into two groups that were different. Females reared on diets containing extracts of PI 503720, PI 505723, CI31A, and PI 485320 were not different from each other in survival to adults but were different from females of the other 11 treatments, which also were not different in survival among themselves (Table 4).

There was an effect of Peruvian maize extracts on fecundity of surviving females (Table 4). No differences were observed in egg fertility among females reared on diets with different Peruvian maize extracts. A high percentage of viable egg masses produced by females grown on diets incorporating extracts shows that the extracts do not affect the female's offspring.

DISCUSSION

Water-soluble compounds from freeze-dried Peruvian maize accessions PI 485320, PI 503720, and PI 503723 had an effect on ECB growth and development. Water extracts of the remaining field-tested insect-resistant accessions had no effect in the laboratory. Lack of activity of some extracts could have resulted from

TABLE 3. LARVAL AND PUPAL DEVELOPMENT TIMES[a] OF ECB WHEN REARED ON DIETS CONTAINING WATER EXTRACTS OF PERUVIAN MAIZE ACCESSIONS, INBREDS WF9 AND CI31A, AND A STANDARD CONTROL DIET

Treatment	Larval development time (days)		Pupal development time (days)		Overall larval and pupal development time (days)	
	Male	Female	Male	Female	Male	Female
PI 503806	15.3 ± 0.3 ab	15.4 ± 0.4 a	7.1 ± 0.3 ab	6.5 ± 0.2 abc	22.4 ± 0.4 a	22.3 ± 0.5 a
WF9 (susc. chk)	15.4 ± 0.4 ab	16.1 ± 0.3 ab	7.6 ± 0.3 ab	6.1 ± 0.2 a	23.2 ± 0.4 ab	22.3 ± 0.4 a
PI 503731	15.1 ± 0.3 ab	15.8 ± 0.3 ab	7.2 ± 0.3 ab	6.4 ± 0.2 ab	22.6 ± 0.5 ab	22.3 ± 0.4 a
Standard Diet	15.0 ± 0.3 a	15.7 ± 0.3 ab	7.5 ± 0.3 ab	6.3 ± 0.2 a	22.9 ± 0.5 ab	22.2 ± 0.4 a
Ames 10623	15.0 ± 0.3 a	16.0 ± 0.4 ab	7.0 ± 0.3 ab	6.2 ± 0.2 a	22.1 ± 0.4 a	22.3 ± 0.5 ab
PI 503727	15.7 ± 0.4 ab	16.7 ± 0.4 ab	6.6 ± 0.4 a	6.4 ± 0.2 ab	22.0 ± 0.6 a	23.1 ± 0.4 ab
PI 503722	15.3 ± 0.3 ab	15.9 ± 0.3 ab	6.9 ± 0.3 a	6.6 ± 0.2 abc	21.9 ± 0.5 a	22.8 ± 0.4 ab
PI 503849	15.4 ± 0.3 ab	15.9 ± 0.3 ab	7.7 ± 0.3 ab	6.5 ± 0.2 ab	23.1 ± 0.4 ab	22.7 ± 0.4 ab
PI 503764	15.8 ± 0.3 ab	16.6 ± 0.4 ab	7.1 ± 0.3 ab	6.6 ± 0.2 abc	23.1 ± 0.4 ab	23.1 ± 0.4 ab
PI 503725	15.5 ± 0.3 ab	16.5 ± 0.3 ab	7.4 ± 0.3 ab	6.7 ± 0.2 abc	23.0 ± 0.4 ab	22.7 ± 0.4 ab
PI 503728	15.4 ± 0.3 ab	16.2 ± 0.3 ab	7.4 ± 0.3 ab	6.6 ± 0.2 abc	23.0 ± 0.4 ab	23.0 ± 0.5 ab
PI 503720	16.0 ± 0.3 ab	16.7 ± 0.3 ab	7.0 ± 0.3 ab	6.9 ± 0.2 abc	23.0 ± 0.4 ab	23.8 ± 0.5 abc
PI 503723	16.7 ± 0.4 b	17.7 ± 0.3 c	7.2 ± 0.4 ab	7.1 ± 0.2 abc	23.9 ± 0.6 ab	24.9 ± 0.5 bc
CI31A (res. chk)	17.0 ± 0.4 b	17.8 ± 0.3 c	9.2 ± 0.6 b	7.7 ± 0.3 c	25.2 ± 0.7 b	26.1 ± 0.5 c
PI 485320	16.7 ± 0.4 b	18.7 ± 0.3 d	8.5 ± 0.7 ab	7.6 ± 0.3 bc	25.2 ± 1.1 ab	25.5 ± 0.6 c

[a]Male and female larval development time, pupal development time, and overall larval and pupal development time are expressed as the means (±SE). Each category was analyzed by ANOVA ($\alpha = 0.05$), and pairwise comparisons within each category were made with an LSMEANS test ($\alpha = 0.05$). Values in each category followed by the same letter indicate means that were not significantly different.

TABLE 4. SURVIVAL OF MALES AND FEMALES, FEMALE LONGEVITY AND FECUNDITY, AND EGG FERTILITY OF ECB REARED ON DIETS CONTAINING EXTRACTS OF PERUVIAN MAIZE ACCESSIONS, INBRED WF9 AND CI31A, AND STANDARD CONTROL DIET[a]

Treatment	Survival of both sexes to the pupal stage [% (*N*)]	Survival from pupal to the adult stages [% (*N*)]		Female longevity (days)	Female fecundity (eggs/female)	Fertility (% viable eggs/female)
		Male	Female			
PI 503806	90.0(27)	83.3(10)a	86.7(12)a	12.3 ± 1.04 a	656.4 ± 36.7 a	91.4 ± 8.3
WF 9 (susc. chk)	96.7(29)	100.0(10)a	100.0(15)a	12.7 ± 0.96 a	635.3 ± 27.5 a	92.0 ± 5.1
PI 503731	100.0(30)	70.0(7)a	85.0(14)a	13.3 ± 0.95 a	551.9 ± 54.0 abcd	80.8 ± 9.8
Standard Diet	90.0(27)	58.3(7)a	93.3(13)a	12.5 ± 0.95 a	539.6 ± 50.5 abcde	85.2 ± 9.3
Ames 10623	90.0(27)	90.0(9)a	100.0(14)a	12.9 ± 1.03 a	587.7 ± 51.2 abc	95.4 ± 4.3
PI 503727	86.7(26)	66.7(6)a	100.0(16)a	14.3 ± 0.99 a	599.0 ± 46.7 abc	96.1 ± 2.5
PI 503722	100.0(30)	70.0(7)a	80.0(16)a	12.7 ± 0.87 a	618.3 ± 32.2 ab	88.1 ± 6.3
PI 503849	93.3(28)	75.0(9)a	93.8(15)a	9.2 ± 0.95 ab	584.7 ± 36.5 abc	99.1 ± 0.9
PI 503764	96.7(28)	75.0(12)a	92.3(12)a	13.1 ± 1.07 a	562.6 ± 62.3 abcd	90.9 ± 8.3
PI 503725	100.0(30)	81.8(9)a	73.7(13)a	12.9 ± 1.01 a	522.9 ± 56.9 bcde	95.3 ± 2.3
PI 503728	96.7(29)	61.5(8)a	62.5(8)a	13.9 ± 1.11 a	633.4 ± 31.7 ab	99.9 ± 0.1
PI 503720	96.7(29)	80.0(8)a	52.6(9)b	11.6 ± 1.15 ab	467.4 ± 98.0 cde	87.3 ± 11.0
PI 503723	96.7(29)	44.4(4)a	50.0(8)b	8.7 ± 1.17 ab	537.6 ± 29.7 abcde	92.0 ± 8.0
CI31A (res. chk)	96.7(29)	40.0(4)a	47.4(9)b	7.2 ± 1.24 b	451.3 ± 60.2 de	98.0 ± 2.0
PI 485320	96.7(29)	10.0(1)b	31.6(6)b	8.6 ± 1.34 ab	405.8 ± 73.0 e	99.8 ± 0.2

[a]Survival was expressed as a percent of the total and analyzed with a chi-square test ($\alpha = 0.05$). Survival to the pupal stage was not significantly different among treatments. Survival from the pupal to the adult stage was significantly different among treatments for both males ($\alpha = 0.05$) and females ($\alpha = 0.05$). Female longevity and fecundity are expressed as the means (± SE). Longevity was analyzed by ANOVA ($\alpha = 0.05$), and pairwise comparisons within each category were made with an LSMEANS test ($\alpha = 0.05$). Fecundity was analyzed by ANOVA ($\alpha = 0.10$), and pairwise comparisons within each category were made with an LSMEANS test ($\alpha = 0.10$). Values in each category followed by the same letter indicate means that are not significantly different. There was no significant difference in fertility among females.

variability in the insects, varying year-to-year field conditions, partial chemical decomposition, metabolism, variability in the solubility of the water soluble compounds making them unavailable to the insect, or the effect as a growth inhibitor may have been influenced by another compound in the accession extract. Studies by Klun et al. (1967) of the water-soluble ECB growth inhibitor DIMBOA showed that it was metabolized from DIMBOA-glycoside, which is present in the intact leaf tissue, and that the growth inhibition caused by DIMBOA could be attenuated by addition of another plant compound, the vitamin niacin, to the diet. Similar chemical processes in Peruvian maize that eliminate or synergistically activate compounds may have mediated efficacy in our study.

The identities of compounds involved in our study have not been determined, but from previous studies of TLC extracts of Peruvian accessions, it was shown that DIMBOA and related compounds were not present in high enough quantities in leaf tissue to account for the observed ECB resistance of maize in the field (Abel et al., 1995). Bergvinson et al. (1995) found by using field-grown plants in a laboratory bioassay that third instars consumed less mature tissue than immature leaf tissue of maize synthetic BS9, despite a high concentration of DIMBOA in the immature tissue. These results led the authors to suggest that larvae are inhibited more by leaf toughness than by the presence of hydroxamic acids (DIMBOA). They noted that while DIMBOA did not seem to act as a feeding deterrent for the third instar, if a longer study encompassing the entire larval stage were done, the results might show negative effects on development and fecundity similar to those observed in the present study and those by Houseman et al. (1992) and Robinson et al. (1978). Numerous observations have demonstrated that maize plants produce and emit a complex mixture of volatile and nonvolatile compounds that are generally recognized as important cues for insect herbivores and parasites that locate and assess host suitability for feeding, refuge, and oviposition (Alborn et al., 1997; Binder and Robbins, 1997; Turlings et al., 1990; Udayagiri and Mason, 1997).

How maize natural products mediate insect behavior and physiology is becoming an intensely studied topic because of the utility of these components in integrated pest management programs (Metcalf and Metcalf, 1992). DIMBOA and related compounds have played a long and important role in the resistance of maize to ECB larvae (Klun and Robinson, 1969; Robinson et al., 1978), but apparently other factors are responsible for the antibiosis in exotic Peruvian germplasm. The possibility of additional factors contributing to ECB resistance was suggested by Bergvinson et al. (1995), who showed that maize biochemicals, such as cell wall phenolic compounds, which are responsible for leaf toughness, correlated negatively with leaf consumption by ECB third instars. The phenolics seemed to augment and help create a semiimpenetrable physical and chemical barrier. Maize silk flavonoids, such as maysin and related compounds, have been proposed as defense elements to prevent attack by larvae of the corn earworm, *Helicoverpa*

zea (Snook et al., 1994). Maize accessions with high concentrations of these compounds were resistant to the corn earworm (Wiseman et al., 1992), and when the compounds were included separately in artificial diets fed to the larvae, all body weights declined in relation to the increasing dietary concentration of the compound (Snook et al., 1994). Like all plants, maize biosynthetic pathways produce a variety of primary and secondary natural products. Many of these, which possibly enhance multiple insect resistance, are not produced in leaf or root tissue in high enough concentrations to affect insects. As shown in our study, only a few genotypes will have high tissue levels. Evidence of the rarity of these genotypes is noted by Abel et al. (1995), who evaluated 1601 accessions from Peru for resistance to the European corn borer and found only 11 lines, representing 0.7% of the total number of evaluations, had qualities worthy of inclusion in a multiple pest resistance breeding program. These accessions express resistant traits to larval feeding and adult oviposition (Abel et al., 1995), and, as the present study shows, novel water-soluble chemical factors other than DIMBOA are produced by these exotic Peruvian accessions. These chemical traits are likely responsible for the deleterious effects on the growth, development, and survival of ECB.

The ECB-resistant Peruvian accessions are descendants of maize populations with high genetic variability, originally collected along the northwest coast of Peru and categorized by Grobman et al. (1961). They belong to four races from geographically distinct regions of Peru and illustrate the importance of searching for new genetic variants that may express qualitative and quantitative traits useful for host plant resistance in North American maize. A few maize lines have already shown resistance to multiple pests, including *O. nubilalis*, *Helicoverpa zea*, *Diatraea saccharalis*, and *Diabrotica* spp. (Davis et al., 1988; Wilson et al., 1995). Identification of chemicals responsible for resistance to ECB and other maize pests from Peruvian germplasm may have importance for maize breeders and entomologists who develop maize lines with durable plant resistance.

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CONTRASTING RESPONSES OF SOUTHERN HOUSE SPIDERS AND RACCOONS TO BLISTER BEETLE PREY

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Abstract—Female southern house spiders, *Kukulcania hibernalis*, readily consumed blister beetles, *Lytta polita*, regardless of cantharidin content when field-tested late in January and again three weeks later in February 1997. In contrast, free-ranging raccoons, *Procyon lotor*, initially ate many *L. polita*, particularly female beetles that contained only one third as much cantharidin as males, but when retested the raccoons ate only a few meloids. These results suggest that raccoons, unlike southern house spiders, quickly form an aversion to blister beetle prey, which is induced by cantharidin. Chemical analyses revealed that southern house spiders ingested 99% of the cantharidin contained within their prey.

Key Words—Cantharidin, chemical defense, Coleoptera, Meloidae, Araneae, Filistatidae, Mammalia, Procyonidae, feeding aversion.

INTRODUCTION

Blister beetles (Coleoptera, Meloidae) are notorious because they contain cantharidin, a noxious substance reputed to possess aphrodisiac properties in preparations called Spanish fly (Keh, 1985; McCormick and Carrel, 1987). Cantharidin is highly toxic to humans and other mammals, causing blistering of skin and similar damage to internal tissues when administered systemically (Till and Majmudar, 1981; Schmitz, 1989; Wang, 1989). Surprisingly little was known about the natural function of this compound until relatively recently, when it was demonstrated that the substance is a potent feeding deterrent to ants, carabid beetles, and some spiders (Carrel and Eisner, 1974; Smedley et al., 1996). Previously we raised the possibility that many avian and mammalian predators might possess oral sensitivity or behavioral aversion to cantharidin, but because

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of its toxicity we refrained from performing the appropriate experiments to test our ideas (Eisner et al., 1990; Yosef et al., 1996).

I here present data, based on experiments in which I offered live meloid beetles to free-ranging spiders and raccoons, showing that the spiders routinely ingest meloids whereas the raccoons evidently learn to reject meloids. To my knowledge, this is the first demonstration of an induced aversion to eating meloids in a vertebrate predator. I also show that the spiders consume virtually all of the cantharidin present within the meloid prey.

METHODS AND MATERIALS

Except for the chemical analyses, which were done at my Columbia laboratory, the experiments were carried out at night at the Archbold Biological Station, Highlands County, Florida, where the animals are nocturnally active.

The meloid beetles, *Lytta polita*, are large, pine-pollen feeding insects (Carrel et al., 1990) and were obtained at night by attracting them with two ultraviolet lights. One "black" light was positioned horizontally beside a conventional 1.2-m-long fluorescent light in a fixture above a white polyester sheet attached on the side of a 1.7-m-tall wooden shelf standing on the cement balcony along the back of the main station building. The other ultraviolet light was placed above a conventional bucket-style insect trap far away from the building, surrounded by typical Florida scrub (Abrahamson et al., 1984). Starting on the evening of January 27, 1997, for four nights in a row I checked the lights hourly between 6 and 11 PM to collect the blister beetles. Meloids (N = 190) were individually put in plastic vials and taken to the laboratory where they were sexed, weighed, and placed in screened cages isolated by sex. Care was taken to prevent reflex bleeding when manipulating the beetles. No beetle was used in more than one trial.

Feeding Trails with Meloid Beetles. Meloids were fed to adult female southern house spiders, *Kukulcania hibernalis* (Araneae, Filistatidae) (N = 20), whose relatively permanent webs were located under the eaves of other station buildings, out of sight of the black lights. These spiders were selected because corpses of *L. polita* are sometimes visible in their webs in winter (Edwards, 1983; Carrel, unpublished). Each spider was presented with either a male or female meloid selected at random. A beetle was flipped into the web from a vial in which it was confined after removal from the cage. Ensuing events were monitored, and the fate of a beetle was recorded. Early season tests were performed at 8–10 PM on January 28–30. They were repeated in the same fashion with the same spiders on February 17–18. Intact carcasses of most eaten beetles, recovered after one to three days when spiders cut the prey from their web and dropped them to the ground, were cleaned of silk, dried for a week at room tem-

perature, weighed, and individually stored at −20°C in clean vials for chemical analysis.

Meloids were also fed to a family of three raccoons, *Procyon lotor* (Mammalia, Procyonidae), who routinely foraged between 8 and 11 PM at the main station building. Having seen the mother and her two offspring eat assorted insects attracted to the illuminated sheet, I decided to determine whether they would attack and eat blister beetles. On one night early in the season (January 29) I immobilized 24 male and 24 female beetles by chilling and then placed them at ~9 PM in a random alignment at 0.1-m intervals on the cement balcony beneath the light. I retreated to monitor the events from a distance of 15 m. I recorded whether the mother or her cubs visibly ate meloids. After the raccoons ambled off, I retrieved the remaining meloids and took them back to the laboratory to check on injury and the sex of surviving individuals.

The tests using the same spiders and raccoons were repeated in a similar fashion three weeks later (February 17 or 18) to ascertain whether their predation behavior had changed. My hypothesis was that the spiders likely would continue to eat meloids, but the raccoons might exhibit a feeding aversion to meloids. Although I could not individually recognize the raccoons, a leading authority on Florida mammals assured me that I almost certainly employed the same raccoons in all of my tests. His reasoning was that it would be virtually impossible for another family unit of the same size to invade this location during my short-term study (J. N. Layne, Senior Research Biologist Emeritus, Archbold Biological Station, personal communication).

Cantharidin Analyses. Cantharidin content of *L. polita* beetles was quantitatively determined by capillary gas chromatography according to a published technique (Carrel et al., 1985). Two types of samples were analyzed: (1) whole, air-dried meloid bodies (15 individuals of each sex), and (2) remains of air-dried meloids after they had been eaten by southern house spiders (15 of each sex).

RESULTS

Predation on Meloids by Spiders

The blister beetles proved very acceptable to southern house spiders (Table 1). In the two feeding trials, only four of 40 meloids given to spiders were not killed and eaten. The spiders showed equal preference to male and female beetles both on the first trial in January and when retested three weeks later in February. Statistical tests revealed no significant effect from the sex of the prey or the time of testing (chi square = 1.11, $df = 1$, $P > 0.1$).

The attack strategy of female *K. hibernalis* was very distinctive. Spiders stealthily moved from their retreats and approached the beetles as they struggled in the cribellate web. After contacting the beetles, or silk near the entrapped

TABLE 1. MELOID BEETLES (*Lytta polita*) EATEN OR UNEATEN BY 20 SOUTHERN HOUSE SPIDERS (*Kukulcania hibernalis*)[a]

	Eaten (*N*)		Uneaten (*N*)	
Trial	Male	Female	Male	Female
First	9	8	1	2
Second	10	9	0	1

[a]Each spider was given one beetle of the same sex in each trial. The intertrial interval was three weeks.

insects, briefly with palps and the front legs (Figure 1A), spiders bit only the tips of the nearest legs or antennae with their small chelicers (Figure 1B), and retreated while their venom took effect. Within 80–300 sec of being bitten, the meloids were immobilized by the spiders' venom. Thereafter the spiders returned, swathed the beetles in silk, and then they began to feed at the head or thorax of the beetles (Figure 1C). None of the attacked meloids reflex bled and there was no visible cleansing of the mouthparts by spiders.

Four beetles managed on their own to struggle free and fall from the webs before being attacked by spiders. This process was slow, ranging from 140 to 320 sec, primarily because the long tarsal claws of meloids repeatedly became entangled in fine loops of silk.

Predation on Meloids by Raccoons. The results with raccoons were in sharp contrast to those with spiders. Of a total of 96 meloids presented in the two trials, only 32 were eaten by the three raccoons. In the first trial (January 29), the raccoons ate 29 living meloids (Table 2). However, in this initial trial they showed a distinct preference for female meloids over males when both were presented in equal numbers (chi square with Yates' correction = 12.5, $df = 1$, $P < 0.001$). When retested three weeks later in February, the raccoons ate only three beetles, all of which were females, and they rejected the remaining 45 animals regardless of sex. The decline in raccoon predation on meloids as a function of time was highly significant (chi square with Yates' correction = 36.8, $df = 1$, $P < 0.001$).

In the first trial, all three raccoons were observed to inspect and sniff meloids on the balcony before eating them. Because it was early in the season and the evenings were cool, few large insects other than meloids were to be found at the lights on the balcony. In several instances after eating blister beetles, raccoons wiped their mouths and then shook their heads from side to side. In the second trial, all three raccoons ate many other large insects that had been attracted to the lights, but only the two cubs approached and contacted blister beetles. The mother raccoon climbed onto the wooden shelf, picked an unidentified katydid off the sheet with her forepaws, and put it in her mouth before

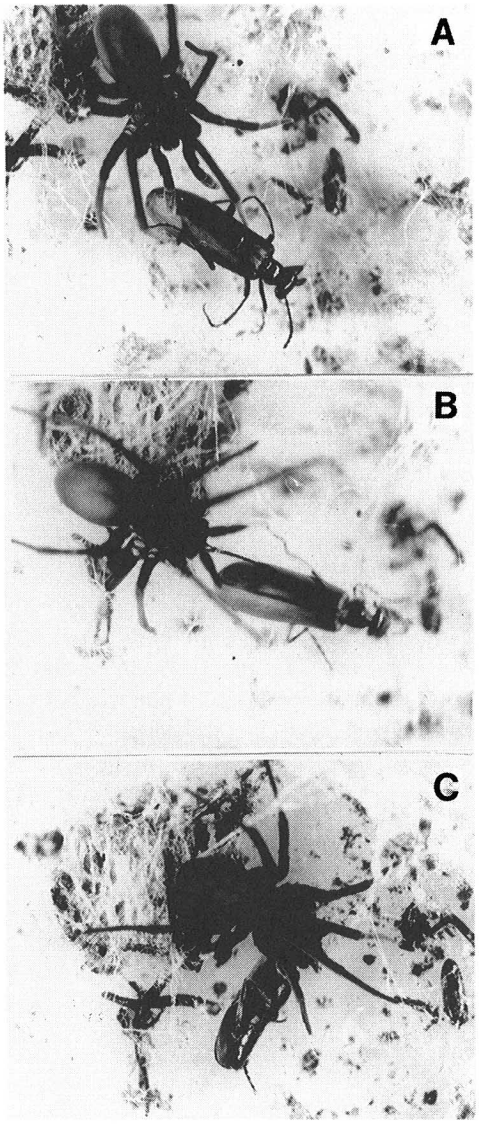

FIG. 1. Three consecutive stages in the attack of a southern house spider (*Kukulcania hibernalis*) on a meloid beetle (*Lytta polita*). (A) The spider uses its pedipalps and first pair of legs to gently contact the beetle struggling in its web. (B) The spider bites only the tarsus on the beetle's left hindleg and injects its paralytic venom before retreating from its prey. (C) Soon after immobilization is complete, the spider returns to feed in the cephalic region of the beetle.

TABLE 2. MELOID BEETLES (*Lytta polita*) EATEN OR UNEATEN BY FAMILY OF THREE RACCOONS (*Procyon lotor*).[a]

	Eaten (*N*)		Uneaten (*N*)	
Trial	Male	Female	Male	Female
First	8	21	16	3
Second	0	3	24	21

[a]Raccoons were given 24 beetles of each sex in each trial. The intertrial interval was three weeks.

she resumed ingesting crunchy scarab beetles and mole crickets. After the raccoons departed, I found two dead female meloids wet with raccoon saliva and 43 unharmed meloids remaining on the balcony.

Cantharidin Analyses. Whole blister beetles contained much cantharidin (Table 3). Male meloids had three times as much cantharidin as females, even though females weighed nearly twice as much as males. Hence, the concentration of cantharidin in *L. polita* males (about 1.1% of dry weight) was about five times higher than that detected in females (about 0.2% of dry body mass).

Remains of blister beetles eaten by southern house spiders weighed considerably less than uneaten beetles (Table 3). When cut into fragments in preparation for extraction of cantharidin residues, I noticed that each beetle's remains consisted of an integumental shell largely devoid of fat body, gut, muscle, and reproductive tissues. The spiders had assimilated nearly two thirds of the dry mass of meloid prey, including virtually all of the cantharidin in their bodies.

TABLE 3. MASS AND NET CANTHARIDIN CONTENT OF UNEATEN BLISTER BEETLES (*Lytta polita* AND REMAINS OF BLISTER BEETLES EATEN BY SPIDER (*Kukulcania hibernalis*)[a]

	Dry mass (mg)	Cantharidin content (μg)
Whole bodies		
Male (*N* = 15)	56.4 ± 3.3 b	627 ± 104 a
Female (*N* = 15)	110.8 ± 11.1 a	204 ± 34 b
Remains		
Male (*N* = 15)	35.4 ± 3.2 c	1.5 ± 0.6 c
Female (*N* = 15)	68.7 ± 5.6 b	0.6 ± 0.2 c

[a]Values are given as mean ± SE. Values sharing the same letter in each column are not significantly different ($P > 0.05$, Tukey HSD test).

DISCUSSION

Like most predators, spiders exhibit a wide range of sensitivities to blister beetles as prey. For example, recent studies performed in central Florida show that the orb-weaver, *Nephila clavipes*, rejects *L. polita* and many other meloids, whereas a sympatric orb-weaver, *Argiope florida*, readily feeds on meloids (Smedley et al., 1996). Crystalline cantharidin is an effective feeding deterrent to *N. clavipes;* hence, the unacceptability of meloids to this spider can be attributed, at least in part, to cantharidin. Although I did not assess the deterrency of pure cantharidin in my tests, the fact that southern house spiders from the same locality ingested several hundred micrograms of cantharidin along with the internal tissues of *L. polita* strongly suggests that this substance is not a potent antifeedant to them.

Systemic absorption of cantharidin from meloid prey may be of mixed adaptive value to spiders. The most obvious risk to the spider is tissue damage caused by cantharidin-induced inhibition of protein dephosphorylation necessary for regulation of cellular activities (Eldridge and Casida, 1995; Liu et al., 1995), which could prove fatal, but it is highly likely that some spiders, frogs, and other predators cope with meloid prey by having cantharidin-resistant protein phosphatases in their cells. A secondary risk, as suggested by Smedley et al. (1996), is that cantharidiniphilic insects, such as pyrochroid beetles and ceratopogonid flies, might be attracted to spiders and bite them in order to obtain this substance for their own use. On the other hand, dietary acquisition and systemic retention of cantharidin might protect spiders from some of their own enemies. For example, one wonders whether the wasp *Allochares azureus* (Hymenoptera: Pompilidae) would attack cantharidin-laden southern house spiders or if its parasitoid larvae would be able to grow and develop as they normally do on this spider (Deyrup et al., 1988). Experiments designed to test these hypotheses are in progress.

The strategy of biting the tips of appendages on insect prey in its web is an unusual, but highly effective mode of attack for a spider. Not only does the spider deliver its venom into the insect's circulating hemolymph, enabling the spread of paralysis throughout the body, but it also protects the spider from retaliatory bites of its prey. Our studies demonstrate that the southern house spider performs this behavior when given other large insects having long appendages, even prey known to be devoid of chemical defenses (J. E. Carrel, in preparation).

Avoidance of *L. polita* by raccoons seems to be a discrimination based on direct experience with the beetles. Moreover, it is reasonable to attribute the raccoons' aversion to meloids to the presence of cantharidin in them, considering that male beetles having much cantharidin were avoided more often than female beetles, which were morphologically very similar but had relatively little cantharidin. The aversion was specific for meloids rather than a general malaise, for

the raccoons ate scarab beetles and other insects attracted to the lights, much as one would expect them to do. In fact, when the tests are performed late in winter, raccoons typically are experiencing food scarcity, hunger, and weight loss because the natural supply of nutritious fruits has been depleted and they subsist on a meager diet of beetles and other insects (Johnson, 1970).

The mechanism whereby omnivorous raccoons develop an aversion to meloids may be varied and complex. For example, after handling or chewing meloids, within hours a raccoon may develop cantharidin-induced blisters on the skin of the forepaw or mouth, resulting in activation of nociceptive neural pathways (Simone et al., 1993) and associative learning. Alternatively, a naive raccoon may quickly form a conditioned food aversion associated with delayed ill effects following ingestion of cantharidin-laden meloids, much as happens in many vertebrates after they have eaten toxic food (Brower and Fink, 1985; Frutos et al., 1997; Kyriazakis et al., 1997; Garcia and Riley, 1998). Of course social learning of one sort or another may also influence diet selection in raccoons, much as it does in rats and some other animals (Galef, 1988). Although few formal studies of learning in raccoons apparently have been performed (Johnson, 1970, and references therein; Conover, 1989), all published results indicate that these mammals possess a remarkable ability to learn and remember, which would explain their adaptability as generalist predators.

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(*Z,Z,E*)-3,6,8-DODECATRIEN-1-OL, A MAJOR COMPONENT OF TRAIL-FOLLOWING PHEROMONE IN TWO SYMPATRIC TERMITE SPECIES *Reticulitermes lucifugus grassei* AND *R. santonensis*

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Abstract—Trail-following bioassays show that trails of the two subterranean termites, *Reticulitermes lucifugus grassei* and *R. santonensis*, which are sympatric in some areas of southwestern France, are not species specific. Even when tested just above threshold level, trails from pentane extracts of whole workers of *R. santonensis* are always preferred by both species. If the *R. santonensis* extract is progressively diluted, the preference is lost. In purified extracts of workers of *R. lucifugus grassei* that elicited a trail-following response, only one compound is active. It was identified by GC-MS as the same major compound of the trail-following pheromone of *R. santonensis:* (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH). The threshold concentration of activity of synthetic DTE-OH was determined to be 10^{-3} ng/cm of trail; optimal activity was obtained at 10^{-2} ng/cm of trail. The increase of trail-following activity of worker extracts of *R. lucifugus grassei* after hydrolysis by potassium hydroxide suggests that DTE-OH also is bound to other components in sternal gland secretions. DTE-OH was also identified in alates of *R. lucifugus grassei*, suggesting that the compound functions both as a trail-following and a sex pheromone, as has been shown to be the case in *R. santonensis*. This demonstrates the high economy developed by termites in their strategies of chemical communication.

Key Words—Trail-following pheromones, (*Z,Z,E*)-3,6,8-dodecatrien-1-ol, specificity, termites, *Reticulitermes lucifugus grassei*, *Reticulitermes santonensis*, GC-MS.

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INTRODUCTION

In termites, chemical communication plays a key role in the regulation and the integration of most of their activities. However, only a few pheromones have been identified in these social insects (Pasteels and Bordereau, 1998). Since the end of the nineteenth century termites have been known to forage along odorous trails (Dudley and Beaumont, 1889), but only two compounds have been identified as trail-following pheromones. (*Z*,*Z*,*E*)-3,6,8-Dodecatrien-1-ol (DTE-OH) was first identified in *Reticulitermes virginicus* (Matsumura et al., 1968; Tai et al., 1969) and then in other Rhinotermitidae, *R. speratus* (Yamaoka et al., 1987) and *Coptotermes formosanus* (Tokoro et al., 1989). (1*E*,5*E*,9*E*)-Neocembrene is the trail-following pheromone of *Nasutitermes exitiosus* (Birch et al., 1972; Kodama et al., 1975) and *Trinervitermes bettonianus* (McDowell and Oloo, 1984). These results raise the question of how specific termite pheromones are and how termite societies remain isolated.

In our study we were interested in the two species of European Rhinotermitidae that are sympatric in some areas in France, *Reticulitermes santonensis* Feytaud and *R. lucifugus grassei* Clément. *R. santonensis* is distributed in western France and *R. lucifugus grassei* in southwestern France. These species coexist around Rochefort and Bordeaux (Clément, 1977). Although the specific status of these termites was controversial in the past (Lash, 1952; Buchli, 1958), different studies have shown separation between *R. lucifugus*, which originally lived in southern France, and *R. santonensis*, which may have been introduced into western France from the United States (Feytaud, 1924, 1925; Clément, 1978, 1979; Bagnères et al., 1990, 1991; Vieau, 1993). From a biometrical study on European species of *R. lucifugus*, Clément (1978) distinguished four subspecies and named *R. lucifugus grassei* the subspecies living in southwestern France.

The aim of our study was to compare the trail-following pheromones of the two species *R. santonensis* and *R. lucifugus grassei*. The trail pheromone of *R. santonensis* was previously identified as DTE-OH (Laduguie et al., 1994a). Here we report the identification of the trail-following pheromone of *R. lucifugus grassei*.

METHODS AND MATERIALS

Biological Material. Species determinations were made according to Clément (1978) and Vieau (1993). Colonies of *R. lucifugus grassei* Clément were collected in stumps of pine wood and infested abandoned huts in southwestern France (Bordeaux, Carcans, Lacanau). Colonies of *R. santonensis* were collected in western and southwestern France (Les Sables d'Olonne, Ile d'Oléron, Saint Jean de Monts, Saint Médard). Colonies of both species were collected in

November 1995 and May 1996 and reared in the laboratory at 25°C, 80% humidity, and a 12L : 12D period. Alates were collected from laboratory colonies and in the fields as mentioned above.

Preparation of Termite Extracts. Workers were separated from colonies and held for two days on moistened filter paper to remove intestinal contents. Individuals were counted and weighed before extraction. The average weight of the extracted workers was 3.09 mg for *R. lucifugus grassei* and 3.60 mg for *R. santonensis*.

For bioassays 50 workers of *R. lucifugus grassei* or *R. santonensis* were extracted for 30 min in 1 ml purified pentane at room temperature. For chemical analyses workers were extracted using a Soxhlet apparatus for 48 hr in purified pentane. The extracts were concentrated to about 1 ml by the Kuderna-Danish method (Marquardt and Luce, 1961) and stored at −20°C for further use. Whole alates were extracted for 48 hr at 4°C with purified pentane.

Treatment with KOH. Because the trail pheromone could be bound or complexed to other compounds, methanolic extracts of *R. lucifugus grassei* were hydrolyzed with KOH before testing. Workers (500) were extracted for 24 hr with methanol in a Soxhlet apparatus. Then the extract was divided in two parts. KOH (2% w/v) was added to one part and 2% (v/v) distilled water was added to the other part as a control. After an 8-hr incubation at room temperature, the methanolic samples were extracted three times with purified pentane. The extracts were pooled, concentrated, and tested for activity in releasing trail-following in a choice bioassay.

Purification and Fractionation of Termite Extracts. Pentane extracts of whole individuals were purified by using either silica gel columns (workers) or high-performance liquid chromatography (HPLC) (alates). In the first method, 3.5 g of purified silica gel (SiO_2 60, 230–400 mesh ASTM, Merck) was added to a glass column (diameter: 10 mm) and successively washed with pentane, dichloromethane, and methanol until no peaks could be detected in the last eluted fraction of each solvent when analyzed by GC. The concentrated worker extract (1.5 ml), corresponding to about 1000 individuals, was loaded onto the top of the silica gel column and developed with 4 × 2 ml pentane, 8 × 2 ml dichloromethane, and 4 × 2 ml methanol. In the second method the extracts were purified by HPLC (Waters apparatus, Waters SiO_2 column, 25 cm × 4 mm ID, 5-μm particle size Lichrospher Si60). An isocratic elution was made using distilled dichloromethane containing 1% (v/v) methanol at a flow rate of 1 ml/min. The UV detector was calibrated at 240 nm. Fractions eluted were collected every 2 min. The signal was recorded and integrated on a computer fitted with PC software (Millenium Waters). The potential biological activity of each fraction obtained by separation on the SiO_2 column or HPLC was tested in a Y-choice bioassay as described below.

Chemical Analysis. GC analyses were carried out with a Chrompack CP-

9002 instrument fitted with a split-splitless injector and a flame-ionization detector. A CP Wax 58 CB (25 m × 0.25 mm ID, 0.22 μm film thickness, Chrompack) fused silica capillary column was used for analyses with temperature programming from 40°C to 240°C, at 2°C/min. Helium was used as carrier gas at a flow rate of 5 ml/min. The temperature of injector and detector was set to 250°C and 270°C, respectively. Collection of the data was done automatically by PC software (Maestro, Chrompack).

GC-MS was conducted with a Nermag R 10-10 C quadrupole mass spectrometer coupled with a Hewlett Packard HP 5890 gas chromatograph. The GC conditions were the same as those described above. The column was connected directly to the ion source of the spectrometer through a heated transfer line maintained at 260°C. Electron impact mass spectra were obtained at 70 eV with a 0.8-sec cycle, and with the instrument scanning from 25 to 300 amu with a source temperature of 150°C.

(*Z,Z,E*)-3,6,8-Dodecatrien-1-ol (DTE-OH) was identified by comparing its spectrum to previously published spectra (Yamaoka et al., 1987; Bordereau et al., 1991). Correct identification was checked by analyzing the biological peak and its corresponding synthetic compound under the same conditions on a CP Wax 58 CB column. Synthetic DTE-OH was a gift from Professor Yamaoka, Institute of Technology, Kyoto, Japan. It was purified by preparative capillary gas chromatography as described by Laduguie et al. (1994a).

Y-Choice Bioassay. Trail-following assays were performed by Y-choice bioassay on Whatman No. 1 filter paper, with a 120° angle between each branch. On the base of the Y (3 cm) and on one of the branches (7 cm), an artificial trail was drawn with a microliter Hamilton syringe containing 1 μl of extract per centimeter of trail. Pure solvent as a control for an activity test or another extract for a choice test was deposited in the same conditions on the Y base and on the other branch. One termite was gently deposited from a small perspex vial onto the Y base. For an activity test, the distance covered by each individual was measured. The maximal response was 10 cm distance, and the activity threshold was defined as a trail followed longer than 3 cm. Results were analyzed with a *t* test ($P < 0.05$). For a choice test, the number of termites that chose one trail was recorded and the data were compared by a Monte Carlo test procedure (Vaillant and Derridj, 1992) based upon chi-square at level $P = 0.05$. Before each test series, concentrated solvent was tested to exclude any trail-following activity of the solvent. For every test a new individual and a new filter paper were used to prevent behavioral conditioning or trail reinforcement.

RESULTS

Trail-Following of R. lucifugus grassei *and* R. santonensis *Workers.* The threshold and maximum activity was first determined for homospecific extracts

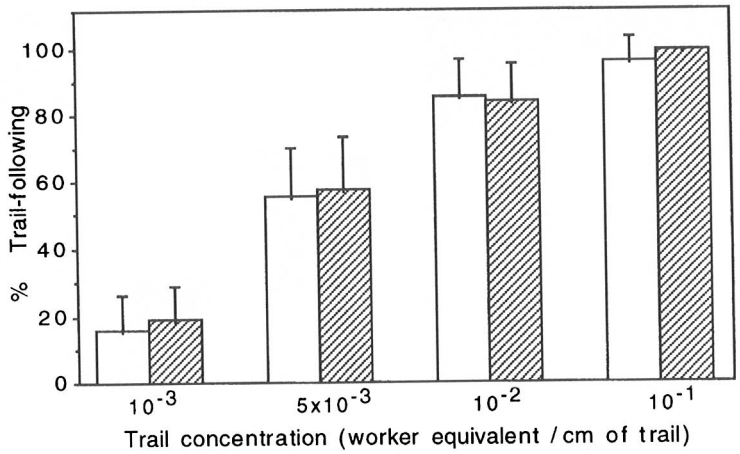

FIG. 1. Trail-following of workers of *Reticulitermes lucifugus grassei* (open bars) and *R. santonensis* (hatched bars) on homospecific trails made from pentane extracts of whole workers. Results are similar in both species, the threshold level being at 5×10^{-3} worker equivalent/cm of trail (mean ± SE); $N = 20$ for 10^{-3} and 10^{-1} concentrations, $N = 30$ for 5×10^{-3} and 10^{-2} concentrations.

of workers (Figure 1). For both species, the activity threshold of whole worker extracts was 5×10^{-3} worker equivalent/cm (WE/cm) of trail, and the maximum activity occurs at 10^{-1} WE/cm of trail.

Choice bioassays between *R. lucifugus grassei* and *R. santonensis* trails were then performed from the threshold concentration to the optimal concentration. At each concentration, workers of *R. lucifugus grassei* (Figure 2A) preferred trails from *R. santonensis* extracts. Results were significantly different for 5×10^{-3} and 10^{-2} WE/cm of trail, not for 10^{-1} WE/cm. Workers of *R. santonensis* (Figure 2B) also preferred trails from *R. santonensis*. The results for *R. santonensis* were statistically significant at each concentration.

The same bioassays were performed after progressive dilution of the extracts of *R. santonensis*. Figure 3A shows that at 5×10^{-2} WE/cm of trail, trail-following of *R. lucifugus grassei* workers was equal on trails made with extracts of *R. lucifugus grassei* or *R. santonensis*. Further dilutions to 3×10^{-2} and 10^{-2} WE/cm of trail resulted in trail-following of 72% and 100%, respectively, for the *R. lucifugus grassei* extracts. Figure 3B shows that after dilution of the extracts of *R. santonensis* to 5×10^{-2} WE/cm of trail, only 56% of their own extracts were followed by *R. santonensis* workers. After further dilution, 29% of *R. santonensis* workers followed their own trail at 3×10^{-2} WE/cm. At 10^{-2} WE/cm of trail of *R. santonensis* extract, only the trails of *R. lucifugus grassei* extract were followed, i.e., the preference for the *R. santonensis* trail was

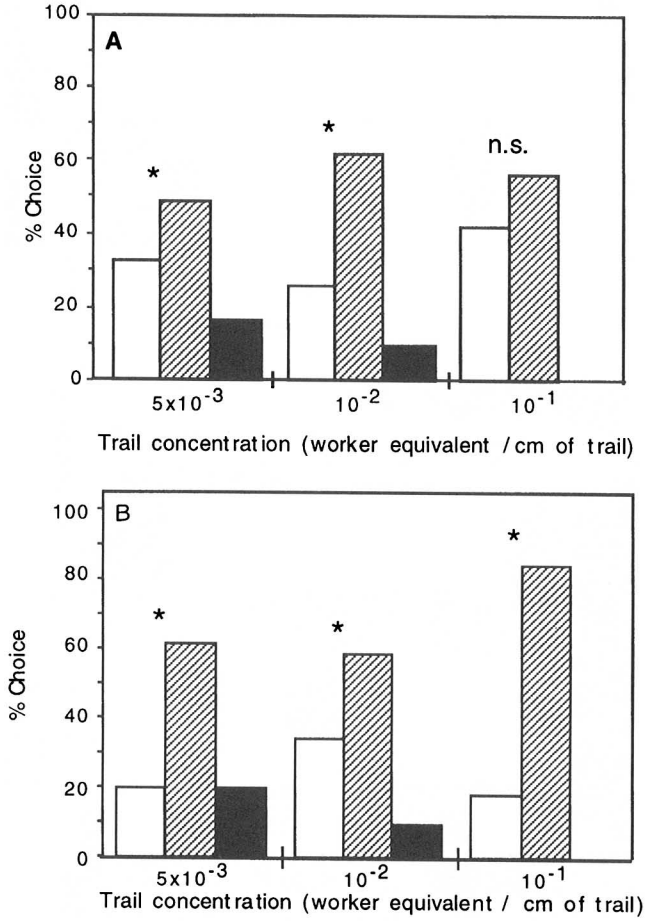

FIG. 2. (A) Choice bioassays for *Reticulitermes lucifugus grassei* workers between equally concentrated trails of *R. lucifugus grassei* (open bars) and *R. santonensis* (hatched bars). Black bars represent the percentage of no choice. Workers of *R. lucifugus grassei* have a significant preference for *R. santonensis* trails, except at the concentration of 10^{-1} WE/cm of trail. *Significant difference at $P < 0.05$, n.s.: no significant difference (Monte Carlo test). $N = 30$ for 5×10^{-3} and 10^{-2} concentrations, $N = 84$ for 10^{-1} concentration. (B) Choice bioassays for *Reticulitermes santonensis* workers between equally concentrated trails of *R. lucifugus grassei* (open bars) and *R. santonensis* (hatched bars). Black bars represent the percentage of no choice. Workers of *R. santonensis* have a significant preference for their own trails at each concentration. *Significant difference at $P < 0.05$, n.s.: no significant difference (Monte Carlo test). $N = 30$ for 5×10^{-3} and 10^{-2} concentrations, $N = 62$ for 10^{-1} concentration.

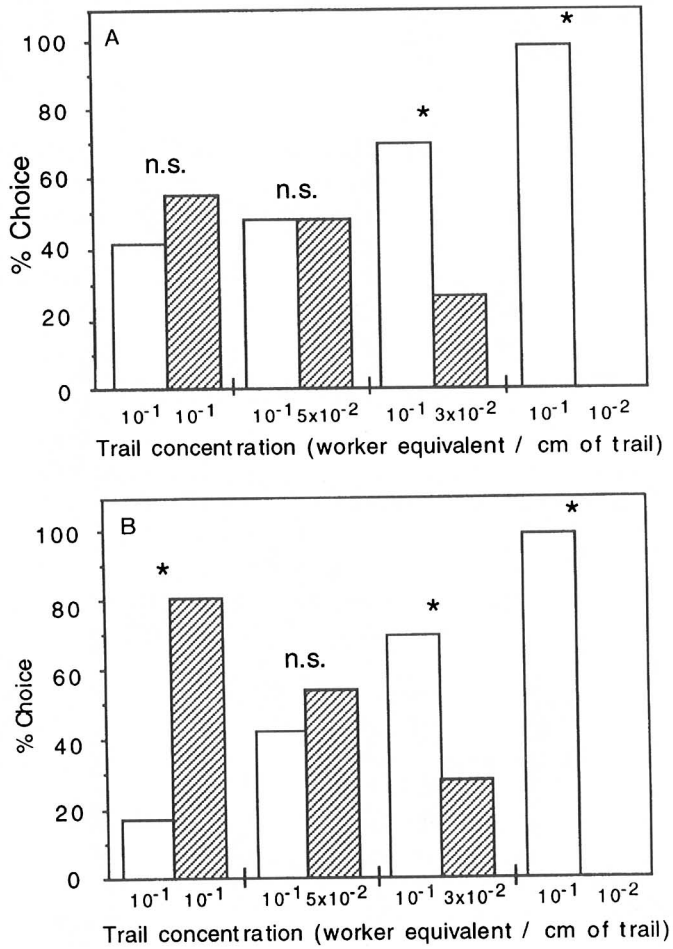

FIG. 3. (A) Choice bioassays for *Reticulitermes lucifugus grassei* workers between trails of *R. lucifugus grassei* (open bars) and *R. santonensis* (hatched bars) after progressive dilution of *R. santonensis* trails. Trails of *R. lucifugus grassei* are significantly preferred when *R. santonensis* trails are 3–10 times less concentrated. *Significant difference at $P < 0.05$, n.s.: no significant difference (Monte Carlo test). $N = 84$ for concentration 10^{-1}, $N = 30$ for the other concentrations. (B) Choice bioassays for *Reticulitermes santonensis* workers between trails of *R. lucifugus grassei* (open bars) and *R. santonensis* (hatched bars) after progressive dilution of *R. santonensis* trails. Preference for *R. santonensis* trails is replaced by preference for *R. lucifugus grassei* when *R. santonensis* trails are diluted 3–10 times. *Significant difference at $P < 0.05$, n.s.: no significant difference (Monte Carlo test). $N = 62$ for 10^{-1} concentration, $N = 30$ for the other concentrations.

changed after diluting the extracts. Both species preferred trails of *R. lucifugus grassei* extract when a 10-times diluted extract of *R. santonensis* was used. From the data of Figure 3B, the trail-following activity of the *R. santonensis* extracts appeared twice as high as that of *R. lucifugus grassei*.

Trail-Following Pheromone of R. lucifugus grassei *Workers*. The crude extract of 1400 workers was purified and fractionated with a silica gel column. Only the last three fractions eluted with dichloromethane were active in trail-following bioassays, and these fractions were investigated by GC-MS. The major peaks were organic acids and alcohols (Figure 4A): octanol, heptadecanoic acid, 9-octadecenoic acid, 9,12-octadecadienoic acid, and two carbonic acid esters—tetradecanoic acid-methylester and octadecanoic acid-methylester. Bioassays of trail-following performed with corresponding synthetic compounds showed that none of these substances elicited trail-following. MS analysis of the small peak that has the same retention time as the synthetic DTE-OH (peak 2, Figure 4B) gave a molecular ion of m/z 180 and fragment ion peaks characteristic for (*Z,Z,E*)-3,6,8-dodecatrien-1-ol.

Synthetic DTE-OH was highly active on workers of *R. lucifugus grassei* and *R. santonensis* in eliciting trail-following, and both species have similar sensitivity to this alcohol (Figure 5). The threshold concentration was determined at 10^{-3} ng/cm of trail and the optimum at 10^{-2} ng/cm. Higher concentration led to lower trail-following activity.

To find out whether in termites DTE-OH was bound to proteins or complexed to another molecule, extracts of workers were hydrolyzed by KOH, extracted with pentane, and tested in a Y-choice bioassay at the concentration of 1 WE/cm of trail. Results obtained clearly showed that extracts were much more active in eliciting trail-following after hydrolysis. In nearly all cases (29 of 30 bioassays) workers of *R. lucifugus grassei* preferred the trails of the hydrolyzed extracts over nonhydrolyzed extracts.

Trail-Following Activity of Alate Extracts of R. lucifugus grassei. Crude extracts of 1600 males and 1600 females of *R. lucifugus grassei* were fractionated by HPLC. The resulting fractions were eluted with dichloromethane and tested in a trail-following bioassay with *R. lucifugus grassei* workers. For male and female extracts, only the fraction eluting between 32 and 38 min in the HPLC run was found to be active for trail-following. DTE-OH was identified in this fraction by GC-MS.

DISCUSSION

GC-MS analysis showed the presence of (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH) in *Reticulitermes lucifugus grassei* workers exclusively fed on filter paper for 48 hr. Of all compounds identified in purified fractions, only DTE-

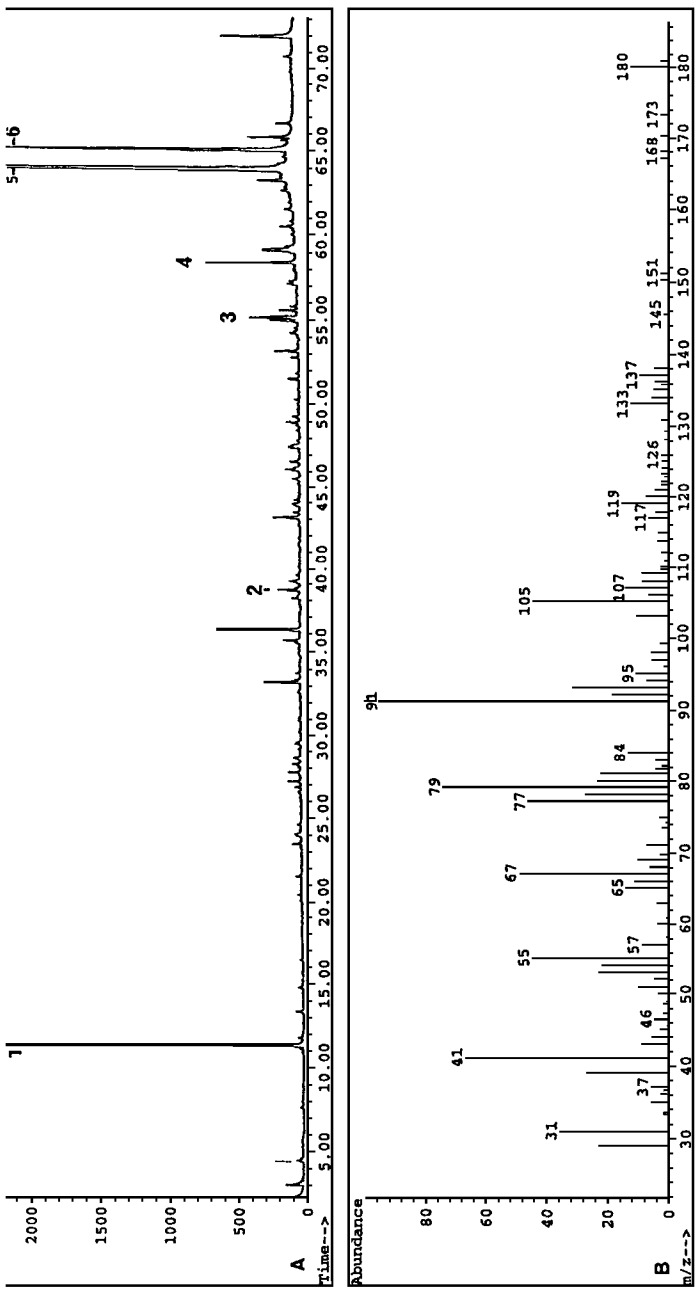

FIG. 4. (A) Gas chromatogram of the active fraction of *R. lucifugus grassei* workers. Peak 1: octanol; 2: (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH); 3: tetradecanoic acid; 4: hexadecanoic acid; 5: 9-octadecenoic acid; 6: 9,12-octadecadienoic acid. (B) EI mass spectrum (70 eV) of compound 2 of a worker extract of *R. lucifugus grassei* identified as (*Z,Z,E*)-3,6,8-dodecatrien-1-ol.

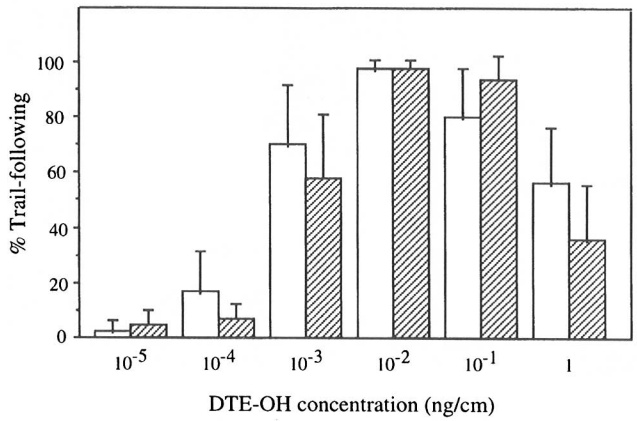

FIG. 5. Trail-following activity of synthetic (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol in workers of *R. lucifugus grassei* (open bars) and *R. santonensis* (hatched bars). No significant difference is observed between *R. santonensis* and *R. lucifugus grassei* responses; both species show a similar sensitivity to this compound. The threshold level is observed at 10^{-3} ng/cm, the optimal activity at 10^{-2} ng/cm. Higher concentrations reduce trail-following. Values are mean ± SE, $N = 30$ for each concentration.

OH was active in eliciting trail-following. Workers of *R. lucifugus grassei* were sensitive to synthetic DTE-OH at pheromonal concentrations. Thus, DTE-OH could be considered the major compound of the trail-following pheromone in *R. lucifugus grassei*.

The same compound was identified as the trail pheromone in several species of Rhinotermitidae; *R. virginicus* (Matsumura et al., 1968, 1969), *R. speratus* (Yamaoka et al., 1987), and *Coptotermes formosanus* (Tokoro et al., 1989). Interestingly, DTE-OH is also the major component of the trail-following pheromone of *R. santonensis* (Laduguie et al., 1994a), which is sympatric with *R. lucifugus grassei* in some areas of France. This could explain why no specificity was observed in choice bioassays of trail-following between both species, even when extracts were tested just above threshold level. Lack of trail-following specificity was also found by Oloo and McDowell (1982) in several species of African termites of the genus *Trinivervitermes* in which the major compound of the trail pheromone was identified as neocembrene (McDowell and Oloo, 1984).

In contrast, Howard et al. (1976) obtained species-specific responses in three species of the subterranean termite *Reticulitermes*. Kaib et al. (1982) showed specific trail-following in *Trinervitermes bettonianus* and *T. gratiosus*.

In some cases, however, the choice of trails could be easily explained by quantitative differences of secreted pheromone between each species. In *R. santonensis* and *R. lucifugus grassei*, for example, the preference for *R. santonen-*

sis extracts was reduced progressively to that of nonpreference and then preference for *R. lucifugus grassei* following progressive dilution of the *R. santonensis* extract. According to our bioassays, workers of *R. santonensis* secrete about twice as much DTE-OH as do *R. lucifugus grassei*. Grace et al. (1995) also showed concentration-mediated trail-following responses to extracts containing trail pheromone in different colonies of *R. hesperus*.

Some data suggest, however, that the trail pheromone of *Reticulitermes* species probably is a multicomponent system, as previously claimed from behavioral observations by Kaib et al. (1982) and Traniello (1982) for other species. Although only the fraction containing DTE-OH was active in eliciting trail-following, some of the other compounds in this fraction could not be identified and tested. The estimated quantities of DTE-OH in termites from chemical analysis (1–10 pg) were 2–100 times lower than those eliciting trail-following in the bioassays (20–100 pg, from Figures 1 and 5), which suggests synergistic components. *R. santonensis* workers appeared to be more reactive to *R. santonensis* trails than *R. lucifugus grassei*. This could be due not only to higher pheromonal concentrations, but might hint at additional species-specific components. Finally, the increase in trail-following activity of the worker extracts of *R. lucifugus grassei* after KOH hydrolysis also suggests storage of esterified precursors of DTE-OH in the sternal gland, as previously shown in *Coptotermes formosanus* by Tokoro et al. (1992).

R. santonensis and *R. lucifugus grassei* were considered in the past as two subspecies of *R. lucifugus* (Lash, 1952; Buchli, 1958) and the nonspecificity of their trail pheromones may underline the close phylogenetic relationship of the two species. DTE-OH and neocembrene are used by several species of termites, however, which demonstrates that chemical communication of termites is unusually nonspecific compared to other social insects. Contrary to trail pheromones of ants, which are secreted by many glands and are chemically variable (Hölldobler and Wilson, 1990), trail pheromones of termites are secreted from only one glandular source, the sternal gland. In termite species in which the workers do not go outside the nest to collect food (species with one-piece life type; Abe, 1987), secretions of their sternal gland probably allows them to mark and recognize their environment and to coordinate their activities of excavating. In species that forage outside the nest (species with separate life type; Abe, 1987), termites very often move along subterranean tunnels or galleries on the surface, which may be considered as expansions of the nest and serve as physical guides. In this case, the orientation role of the trail-following pheromone is limited to the first steps in building the galleries. In fact, specific trails only appear useful for termite species foraging above ground in open air and far from the nest. This is infrequent, but does occur in harvester termites such as *Trinervitermes bettonianus* and *T. gratiosus*, in which species-specific trails were demonstrated by Kaib et al. (1982).

DTE-OH was also found in alates of *R. lucifugus grassei*. Probably it is used as a trail-following pheromone in these individuals during the nuptial promenade. Although it could not be tested for its sexual activity, it might represent a major component of the female sex pheromone of this species as previously shown for the sympatric species *R. santonensis* (Laduguie et al., 1994a). Clément (1982) noted that the reproductive isolation of both species would not arise from sex pheromone specificity since males of *R. santonensis* were attracted by females of *R. santonensis* and *R. lucifugus grassei*. The main factor of isolation is the time lag between swarming periods of the two species. In their area of sympatry, the dispersal flights of *R. santonensis* occur at the beginning of April and those of *R. lucifugus grassei* at the beginning of May. These results are similar to those observed in two African fungus-growing termites *Pseudacanthotermes spiniger* and *P. militaris* (Bordereau et al., 1991, 1993; Laduguie et al., 1994b) and show again the economy developed by termites in their strategies of chemical communication.

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EFFECTS OF TANSY ESSENTIAL OIL FROM *Tanacetum vulgare* ON BIOLOGY OF OBLIQUE-BANDED LEAFROLLER, *Choristoneura rosaceana*

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Abstract—The formulations TSW (tansy essential oil, surfactant, distilled water) and TSWD (tansy essential oil, surfactant, distilled water, dillapiol) increased the feeding rate of oblique-banded leafroller (OBLR) larvae with increasing concentrations of tansy essential oil. When tansy essential oil was continuously present at 0.1% and 1% in the diet, all OBLR larvae from susceptible and resistant populations died. The presence of 0.01% tansy essential oil in the diet affected female pupal weight, but not larval weight gain, larval developmental time and male pupal weight. Our results suggest that the concentration of tansy essential oil that significantly affects the developmental variables should be between 0.01% and 0.1% of tansy essential oil. Residues of the formulation TE (tansy essential oil, ethanol 95%) deterred oviposition of OBLR female.

Key Words—Antifeedant activity, larval development, tansy essential oil, oviposition deterrent, *Tanacetum vulgare*, dillapiol, *Choristoneura rosaceana*, oblique-banded leafroller, tansy essential oil residues.

INTRODUCTION

The oblique-banded leafroller (OBLR), *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), is a native polyphagous species widely distributed in temperate North America (Reissig et al., 1986). Larvae preferably feed on leaves

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and fruit of rosaceaous plants, including apple trees (Chapman and Lienk, 1971). Since the 1980s, OBLR populations have developed resistance to synthetic insectidies in orchards of New York State (Reissig et al., 1986), Québec (Carrière et al., 1994; Smirle et al., 1998), and Ontario (David Pree, personal communication). Among the alternative methods to control OBLR populations are *Bacillus thuringiensis* var. *kurstaki* (Li et al., 1995; Li and Fitzpatrick, 1996) and mating disruption with pheromone (Lawson et al., 1996).

Another alternative control strategy is the utilization of botanical insecticides from plant extracts that show broad-spectrum activity against phytophagous insects. For example, neem seed oil has been shown to affect the fitness of OBLR (Lowery et al., 1996). Another plant extract possessing insecticidal properties is tansy, *Tanacetum vulgare* (L.) (Asteraceae), which is found in temperate areas of the northern hemisphere. Russians and Native Amerindians from the Northeast used tansy as an insecticide by spreading their fields with dried extracts of the plant (Chandler et al., 1982). At least 20–30 chemotypes of tansy have been described in the literature (Dembitskii et al., 1984; Holopainen et al., 1987; Nano et al., 1979). They are differentiated mainly by the relative amounts of α- and β-thujone found in their essential oil, with thujone lacking in a few of them. In our experiments, we used tansy essential oil containing an average of 90% of β-thujone (i.e., a β-thujone chemotype), a monoterpene cetone having antifeedant activity against Colorado potato beetle adults, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Panasiuk, 1984; Schearer, 1984). Aqueous extracts of tansy also have antifeedant effects on imported cabbageworm larvae, *Artogeia* (=*Pieris*) *rapae* (L.) (Lepidoptera), diamondback moth larvae, *Plutella xylostella* (L.) (Lepidoptera) (Brewer and Ball, 1981; Hough-Goldstein and Hahn, 1992), Colorado potato beetle adults and larvae, *Leptinotarsa decemlineata* (Say) (Hough-Goldstein, 1990), and codling moth larvae, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Suomi et al., 1986).

Metcalf (1967) suggested that the term synergism refers to situations where one component of a mixture, the synergist, is inactive at the dosage employed and where the mixture is appreciably more active than the other component alone. In our experiment, dillapiol, a phenylpropanoid compound found in the dill plant, *Anethum graveolens* L. (Apiaceae), has been used as a synergist with tansy essential oil. Dillapiol is a derivative of methylenedioxyphenyl (MDP), a chemical group among which several synergists are found and are currently in use (Casida, 1970). Dillapiol synergizes the insecticidal activity of carbaryl, carbofuran, and parathion on fruit flies, *Drosophila melanogaster* Meig., and mosquito larvae, *Aedes aegypti* L. (Lichtenstein et al., 1974), and with pyrethrin and carbaryl, it is active against the adults of the red flour beetles, *Tribolium castaneum* Herbst (Maheshwari et al., 1978; Tomar et al., 1978). Furthermore, in the European corn borer, *Ostrinia nubilalis* Hubner, dillapiol inhibits the polysub-

strate monooxygenase (PSMOs), a group of enzymes that transforms lipophilic compounds into more hydrophilic metabolites (Bernard et al., 1989). Research has been done on the synergistic effect of dillapiol with synthetic insecticides (Saxena et al., 1978) but little information is available on dillapiol as a synergist when mixed with natural insecticides.

Tansy odor had a deterrent effect on oviposition and mating behavior in the European grapevine moth, *Lobesia botrana* Den. and Schiff. (Lepidoptera: Tortricidae), and exposure to tansy odor reduced longevity of adults (Gabel and Thiéry, 1994). When *L. botrana* females were in the presence of tansy essential oil odor, egg laying was reduced by 30–80%. Oviposition by females is particularly important for the survival of their progenies because the quality of food and its availability to larvae is determined by oviposition of the parent female on a suitable host plant (Chapman, 1974; Honda, 1995). For instance, females of the oblique-banded leafroller preferred to oviposit on apple trees (Carrière et al., 1995), and it was demonstrated that females emerging from larvae that had fed on apple leaves had a 26% higher mean fecundity than if fed on trembling aspen, wild rose, or snowberry (Carrière, 1992).

In this paper, three biological properties of tansy oil have been investigated by using OBLR as a model. The goals of our study were as follows: (1) to examine the antifeedant activity of one formulation containing tansy essential oil and a formulation containing tansy essential oil and dillapiol on third instars from a resistant OBLR population; (2) to determine the effects of tansy essential oil on growth and development of resistant and susceptible OBLR populations; and (3) to determine the oviposition deterrent activity of residues of tansy essential oil.

METHODS AND MATERIALS

Insect Rearing. Overwintered OBLR larvae were collected in June 1995. The insecticide-resistant population was collected from a commercial apple orchard in Deux-Montagnes (45°29′N, 74°03′W), Quebec, Canada. The insecticide-susceptible population was collected in an unsprayed forest in Sainte-Foy (46°48′N, 71°13′W), Quebec, Canada. The populations were reared in separate growth chambers (21°C, 16L : 8D photoperiod, 65% relative humidity) on a pinto bean-based artificial diet (modified from Shorey and Hale, 1965). The two populations were reared continuously for three generations.

Plant Materials. Essential oil of *Tanacetum vulgare* was obtained by steam distillation of fresh tansy plants, harvested in September 1995 at Lavaltrie (45°53′N, 73°15′W), Quebec, Canada, in a 380-liter portable distillation unit. The tansy essential oil was analyzed by capillary gas chromatography (GC) and by gas chromatography–mass spectrometry (GC-MS). Gas chromatography was

carried out with a Varian 6000 series Vista chromatograph equipped with two flame ionization detectors. GC-MS analyses were carried out on a Varian Star 3400 gas chromatograph connected to a Varian Saturn II Ion trap mass detector equipped with a PC data system. Peak areas were computed by a Varian DS 654 integrator. Fused silica Durabond DB-1 (0.25-mm film thickness, 30 mm × 0.25 mm ID) and a fused silica Durabond DB-Wax (0.25-mm film thickness, 30 mm × 0.25 mm ID) (J & W Scientific, Folsom, California) capillary column were used for the GC analyses. Fused silica Durabond DB-5 (0.25-mm film thickness, 30 m × 0.25 mm ID) capillary column was used for the GC-MS analyses. Component identification was based on a comparison of spectra with mass spectra of reference substances and an NIST data program, as well as on Kovats' retention indices obtained on two phases of different polarity compared to Jennings and Shibamoto (1980), and Kovats' retention indices obtained on DB-5 and compared to indices from Adams (1995). The oven temperature program used with all capillary columns was initially at 40°C with 2°C/min rise to 250°C. The injector and detector temperatures were set at 230°C and 250°C, respectively. Injections of 1 μl were made in the splitless mode and changed to the split mode after 0.10 min. The tansy essential oil contained 88.6 β-thujone, 6.5% germacrene-D, 1.1% α-thujone, 0.5% *p*-cymene, 0.4% sabinene, 0.4% β-caryophyllene, 0.4% 1,8-cineole, 0.4% camphor, 0.2% 4-terpineole, and 1.5% unidentified. Three formulations containing tansy essential oil and a control were tested against OBLR (Table 1).

Feeding Activity. Antifeedant activities of formulations TSW and TSWD were assayed by using nitrocellulose disks (6.5 mm diameter). Fifteen disks were pinned on a styrofoam tray (22.7 × 28.0 cm). Each disk, set ca. 1 mm from the tray to facilitate larval feeding, received 5 μl of the formulation or a control solution. Sixty larvae (5–25 mg) from the resistant population (10 replicates of six larvae per concentration) were fasted for 24 hr. The insects were individually placed near the treated cellulose disk and covered with a Petri dish lid (3.5 cm diameter). The styrofoam trays were covered with plastic wrap and were placed in a growth chamber at 21°C, 16L : 8D photoperiod, and 65% relative humidity. After 24 hr, larvae were removed from the disk. To measure antifeedant activity, the area of nitrocellulose disks consumed by the larvae was assessed and compared to control disks by using a digital image analyzer (Panneton and Drummond, 1991).

Chronic Exposure. To determine the effect of chronic exposure on the larvae of both populations, tansy essential oil was incorporated in 200 ml of diet at concentrations of 0.01, 0.1, and 1%. Test diets were prepared by thoroughly mixing the essential oil and artificial diet (45°C) with a spoon for 1 min. Diet without added tansy essential oil was used as the control. Newly emerged larvae were transferred individually to plastic cups (ca. 30 ml) containing the diet, and insects were incubated as described above. The experiment was replicated 10

TABLE 1. DESCRIPTION OF THREE FORMULATIONS CONTAINING TANSY OIL

Concentration of tansy oil (%)	Tansy oil (g)	Ethanol 95% (g)	Surfactant (g)[a]	Dillapiol (g)[b]	Distilled water (g)
Formulation 1 (TE)					
10	0.100	0.900			
1	0.010	0.990			
0.1	0.001	0.999			
Control		1.000			
Formulation 2 (TSW)					
10	0.100		0.100		0.800
1	0.010		0.010		0.980
0.1	0.001		0.001		0.998
Control			0.100		0.900
Formulation 3 (TSWD)					
10	0.100		0.100	0.0100	0.790
1	0.010		0.010	0.0010	0.979
0.1	0.001		0.001	0.0001	0.998
Control			0.100	0.0100	0.890

[a]Surfactant: Alkamuls EL-620, Cranbury, New Jersey, in a ratio of 1 : 1 to tansy oil.
[b]Dillapiol, in a ratio of 1 : 10 to tansy oil.

times, with six larvae per concentration. Larval and pupal weights, larval mortality, and rates of pupation and adult emergence were recorded every fourth day for 67 days.

Oviposition Activity. Female and male pupae were reared separately in a mating chamber consisting of a plastic vial (4 × 6.5 cm) whose bottom was removed and replaced by a cap. A small glass containing a cotton roll soaked in 10% sucrose for the emerging adults to feed on was inserted through the cover. The top of the mating chamber was covered with fine muslin held tight by a plastic ring (40 mm diameter). Upon female emergence, two male moths (less than 24 hr old) were transferred to a mating chamber containing a female for 24 hr. For the oviposition assay, 200 μl of formulation TE was coated inside a beaker (50 ml). When the inner surface was dry (2 hr), the beaker was turned over (i.e., opening down), one female was inserted in the beaker, and the opening was covered with a fine muslin. The beaker was incubated in a growth chamber at 21°C, 16L : 8D and 65% relative humidity. After 24 hr, the female was removed from the beaker and the presence of egg masses was noted. The number of eggs in each mass was counted under a binocular microscope. Twenty-five females were bioassayed individually for each concentration.

Statistical Analyses. In the experiment on antifeedant activity, the comparison of disks area consumed of formulation TSW and TSWD were subjected to ANOVA. Survival data from the chronic exposure test were analyzed with contingency tables and multiple comparisons for proportions (Abacus Concepts,

Inc., 1992; Zar, 1984). For each population, larval weight gain, pupal weight, and larval development in chronic exposure test were subjected to ANOVA (Abacus Concepts, Inc., 1992). Linear regression was used to determine the relationship between concentration and total eggs laid.

RESULTS

Feeding Activity. In the TSW formulation, disks treated with 0.1% of tansy essential oil were ($P < 0.05$) less consumed (8.3%) than those with 10% oil (18.5%) and controls (15.9%) (Figure 1A). Concentrations of 1 and 10% tansy essential oil were not significantly ($P = 0.05$) different from control. An increase of tansy essential oil concentration increased larval feeding with formulation TSWD (i.e., a mixture of tansy essential oil and dillapiol) (Figure 1B). Disks treated with 1 and 10% of tansy essential oil were ($P < 0.05$) more consumed than those with 0.1% tansy essential oil and control.

Chronic Exposure. In the susceptible population, OBLR larvae reared for four days on diet containing 1% tansy essential oil had a survival rate (0%) ($P < 0.001$) lower than those reared at 0.1% tansy essential oil (30%) (Figure 2). At concentrations 0.1 and 1%, all larvae were dead after 45 days. Percentage of

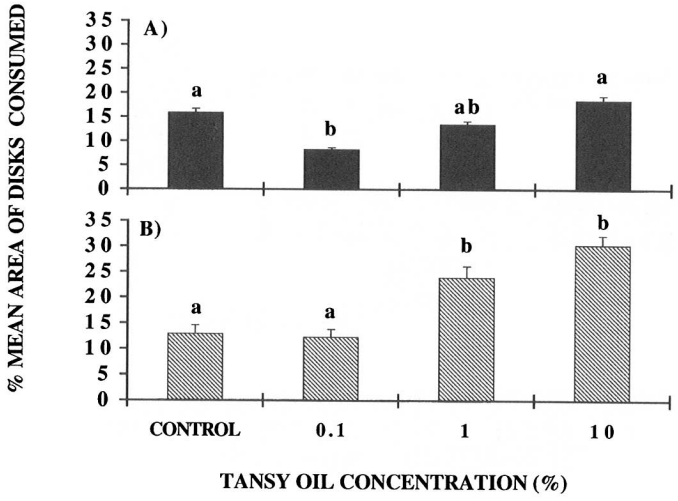

FIG. 1. Mean area of disks treated with (A) the TSW formulation and (B) the TSWD formulation consumed by third instars. A control disk of 6.5 mm diameter = 100%. Bars with the same letters are not different based on ANOVA and Tukey test for separation of means ($P = 0.05$).

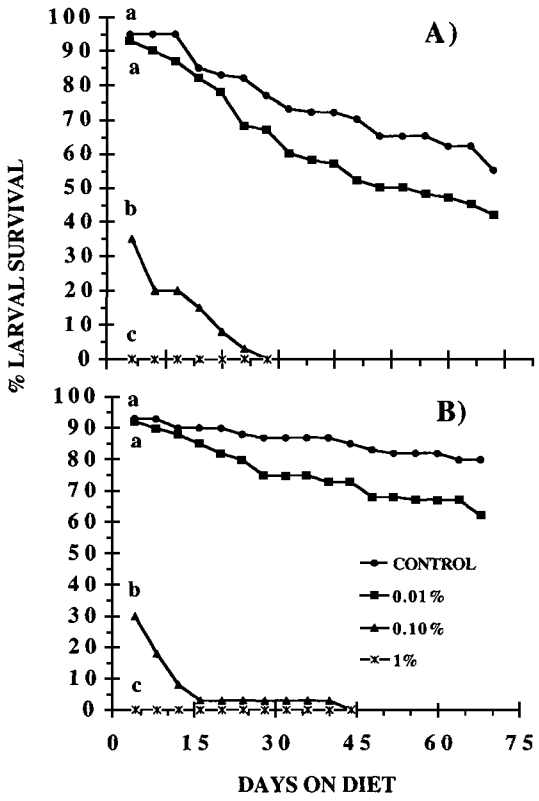

FIG. 2. Survival rate of (A) a resistant population, and (B) a susceptible population reared on diet containing tansy essential oil during 68 days. At day 4, survival rate values followed by the same letters are not significantly different ($P = 0.05$), based on test of proportions (Zar, 1984).

larval survival at 0.1% (30%) was ($P < 0.001$) lower than that at 0.01% (92%) and in the controls (93%) after four days. Larval survival rates, in the controls and 0.01% treatments were higher than 60% throughout the experiment. There was no significant ($P = 0.05$) difference between the control and 0.01% tansy essential oil treatment from days 4 to 68. In the resistant population, no first instars survived on diet containing 1% tansy essential oil after four days (Figure 2). Percentage of larval survival at 1% (0%) was ($P < 0.001$) lower than that at 0.1% tansy essential oil (35%) after four days. After 28 days on diet treated with 0.1 and 1% tansy essential oil, all of the larvae were dead. Larval survival rate at 0.01% (93%) was ($P < 0.001$) higher than that at 0.1% (35%) at day 4, but was not ($P = 0.05$) different than that of control from days 4 to 68.

TABLE 2. DEVELOPMENT OF SUSCEPTIBLE AND RESISTANT OBLR POPULATIONS CHRONICALLY EXPOSED TO TANSY ESSENTIAL OIL IN ARTIFICIAL DIET

Developmental parameter and concentration of tansy oil (%)	Population[a]			
	Susceptible		Resistant	
	Mean ± SE	*N*	Mean ± SE	*N*
Larval weight gain (mg/day)				
0 (Control)	14.8 ± 1.3a	20	14.1 ± 1.6a	16
0.01	11.7 ± 1.4a	14	11.8 ± 1.3a	9
Larval development time (L1-pupa) in days				
0 (Control)	42.2 ± 3.5a	20	38.5 ± 3.3a	16
0.01	41.1 ± 3.6a	14	45.8 ± 4.0a	9
Female pupal weight (mg)				
0 (Control)	90.1 ± 2.9a	14	94.4 ± 6.8a	7
0.01	108.1 ± 3.4b	3	74.6 ± 5.4b	7
Male pupal weight (mg)				
0 (Control)	55.3 ± 2.7a	6	49.6 ± 2.9a	9
0.01	54.6 ± 2.0a	11	56.2 ± 1.2a	3

[a]In each population and for each developmental parameter, values followed by the same letters are not significantly different ($P = 0.05$), based on ANOVA (Abacus Concepts, Inc., 1992).

Because no larvae survived on diet containing 0.1 and 1% tansy essential oil, developmental parameters of the larvae at 0.01% only were compared with the control (Table 2). There were no ($P = 0.05$) differences between control diet and 0.01% for larval weight gain, larval development time, and male pupal weight in both populations. However, in the susceptible population, female pupae from the control were ($P < 0.05$) lighter (90.1 mg) than female pupae reared on 0.01% tansy essential oil (108.1 mg). In the resistant population, female pupae of the control (94.4 mg) weighed more ($P < 0.05$) than female pupae reared on 0.01% tansy essential oil (74.6 mg).

Oviposition Activity. Egg-laying rate on TE formulation residues decreased with increasing concentrations of tansy essential oil (Figure 3). The linear regression between the number of eggs laid on dried residues and the concentration of tansy essential oil in TE formulation was significant ($P < 0.05$, $R^2 = 0.93$). No females survived when exposed to 10% tansy essential oil dried residues.

DISCUSSION

Laboratory tests by Suomi et al. (1986) demonstrated that neonate codling moth larvae, *Cydia pomonella* (L.), ate only 3% of apple cores when treated with 1% of tansy extract. Similar results were obtained in other experiments with

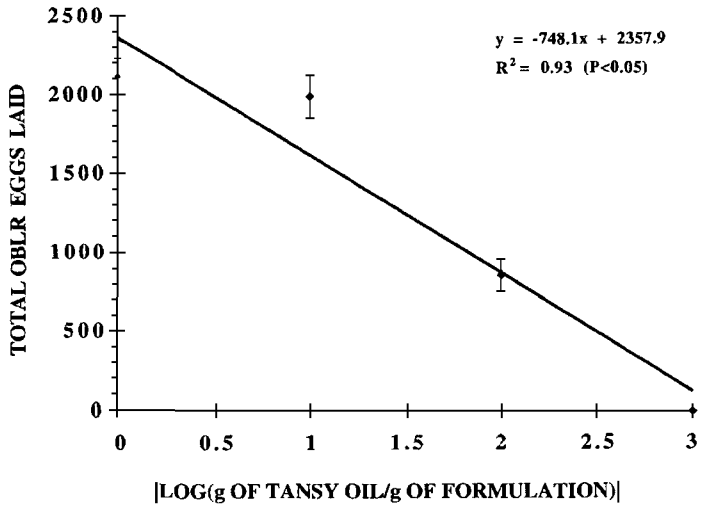

FIG. 3. Number of eggs laid by the OBLR female on dried residues of tansy essential oil (formulation TE).

lepidopteran species, i.e., *P. rapae* and *P. xylostella* (Brewer and Ball, 1981; Hough-Goldstein and Hahn, 1992). Our results show that formulations TSW and TSWD, containing tansy essential oil, have no antifeedant activity toward OBLR larvae, as expected in previous reports (Brewer and Ball, 1981). At 0.1% tansy essential oil, TSW exerted a deterrent effect on OBLR larvae compared to the control. At lower concentrations, only 8% of the 0.1% treated disk area was eaten by the larvae. Schoonhoven et al. (1992) reported that some substances produce higher responses at low concentrations than at higher concentrations.

According to previously reported experiments, dillapiol inhibits the PSMOs detoxification enzymes and, potentially, other mechanisms, leading to resistance. It is of interest to know that it increased the feeding of insects when presented together with tansy essential oil, but this most likely resulted from its effects on the metabolism of food transformation rather than from its interference with feeding behavior. Furthermore, it was shown that dillapiol did not enhance the activity of some insecticidal allelochemicals from Asteraceae when ingested by the European corn borer (Bernard et al., 1990). In this study, the synergistic antifeedant effect of dillapiol was investigated, and the presence of dillapiol with tansy essential oil in formulation TSWD increased the feeding activity by OBLR larvae compared to formulation TSW and the control. To our knowledge, this is the first published report on the phagostimulant effect of tansy essential oil and dillapiol toward an insect species. In this way, dillapiol could be mixed

with natural insecticides such as *Bacillus thuringiensis* Berl. to increase ingestion.

The specific mechanism of action of tansy essential oil on larvae is unknown. However, the toxicity effect of monoterpene vapors could have been in part responsible for OBLR larval mortality after four days of exposure to 0.1 and 1% tansy essential oil in the diet. In a closed area, monoterpene vapors have a fumigant action on adults of several insect species (Smith, 1965; Coats et al., 1991). In our chronic exposure bioassay, larvae were reared in a closed area where the tansy essential oil vapors were likely continually released by the diet. Furthermore, tansy essential oil in the diet could be topically toxic to the larvae by a continuous contact with the cuticular membrane.

Hough-Goldstein and Hahn (1992) reported that larvae of imported cabbageworm reared on leaves treated with 5% of tansy extract took longer to develop and had smaller pupae. In our experiment, chronic exposure to tansy essential oil at 0.01% in the artificial diet did not affect larval weight gain, larval development time, and pupal weight of males in susceptible and resistant populations, but did affect female pupal weight. Monoterpenoids are lipophilic compounds, and they may exert toxic interference with biochemical and physiological functions of insect herbivores (Brattsten, 1983). The concentration that exerts a significant effect on OBLR development should be between 0.01 and 0.1% of tansy essential oil in diet.

Dried residues of TE formulation containing tansy essential oil deterred OBLR female oviposition. Gabel and Thiéry (1994) suggested that physiological and behavioral mechanisms directly related to oviposition can be affected by the presence of tansy odor. In many lepidopterous insects, secondary compound from plants stimulate or inhibit oviposition of females (Honda, 1995).

Laboratory experiments showed that larvae and female pupae from both populations were affected by the presence of tansy essential oil in the diet, and tansy essential oil residues from TE formulation had oviposition deterrent effect on OBLR female. Tansy essential oil has a wide activity spectrum toward several pests as repellents, attractants, growth regulators, and oviposition deterrent but few publications concern its mode of action. Further investigations must be made to identify the monoterpenoids in tansy essential oil responsible for the biological effect on OBLR.

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PHYTOCHEMICAL VARIATION IN QUAKING ASPEN: EFFECTS ON GYPSY MOTH SUSCEPTIBILITY TO NUCLEAR POLYHEDROSIS VIRUS

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Abstract—The performance of gypsy moths (*Lymantria dispar*) feeding on quaking aspen (*Populus tremuloides*) is strongly influenced by host foliar chemistry and susceptibility to a nuclear polyhedrosis virus (LdNPV), but the relationship of susceptibility to chemistry is poorly understood. We investigated the effects of genetic and resource-mediated variation in phytochemistry on viral pathogenicity. Trees were grown in pots in a common garden. Disks were punched from aspen leaves, inoculated with LdNPV and fed to third instars. Additional leaves were analyzed for levels of nitrogen, starch, phenolic glycosides, and condensed tannins. Despite marked variation among trees in levels of phenolic glycosides and tannins, we observed minimal variation in larval susceptibility to LdNPV. Viral pathogenicity was only weakly (inversely) correlated with tannin concentrations in one of two experiments. These results suggest that differential defoliation of aspen by gypsy moths in the field is due to the direct effects of host chemistry on larval performance rather than to the indirect effects of host chemistry on efficacy of this natural enemy.

Key Words—Aspen, genetic variation, gypsy moth, *Lymantria dispar*, nuclear polyhedrosis virus, *Populus tremuloides*, phenolic glycosides, tannins, tritrophic interactions.

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INTRODUCTION

The individual performance and population dynamics of herbivorous insects are influenced by bottom-up and top-down factors and the interplay between them. Historically, bottom-up perspectives have emphasized studies of genetic and resource-mediated variation in plant chemical composition and the consequences for herbivores. Top-down perspectives have emphasized research on factors influencing the efficacy of predators, parasitoids, and pathogens. More recently, the potential for indirect effects of plant chemistry that are mediated by changes in the efficacy of natural enemies on herbivorous insects has been recognized (Price et al., 1980; Foster et al., 1992). Yet, how the efficacy of natural enemies is influenced by genetically or environmentally influenced variation in accumulation of specific plant chemicals remains poorly understood.

One system particularly well suited for investigation of such indirect effects consists of quaking aspen (*Populus tremuloides*), gypsy moths (*Lymantria dispar*), and the gypsy moth nuclear polyhedrosis virus (LdNPV). Aspen comprises a major forest type in the Great Lakes region, where gypsy moths have recently become established. During outbreaks of folivorous insects such as gypsy moths and forest tent caterpillars (*Malacosoma disstria*), aspen trees and clones are differentially defoliated (Lindroth, Hwang, and Osier, personal observation).

Differential defoliation results, at least in part, from the direct effects of foliar chemistry on herbivorous insects. Aspen trees and clones exhibit striking variation in secondary chemistry (phenolic glycosides and condensed tannins), due to both genetic variability and resource availability (Hwang and Lindroth, 1997; Lindroth and Hwang, 1996a; Hemming and Lindroth, 1999). Phenolic glycosides, at moderate to high concentrations, reduce the performance (growth and fecundity) of gypsy moths and other aspen-feeding insects (Hemming and Lindroth, 1995; Lindroth and Hwang, 1996a; Lindroth et al., 1997; Hwang and Lindroth, 1998). Conversely, condensed tannins, with equally variable concentrations in aspen, have little direct effect on folivorous insects (Hemming and Lindroth, 1995; Lindroth and Hwang, 1996a; Hwang and Lindroth, 1998).

Differential defoliation may also result from the indirect effects of host chemistry on viral pathogenicity. Historically, LdNPV has been the principal pathogen contributing to the collapse of gypsy moth outbreaks (Elkinton and Liebhold, 1990). LdNPV played an important role in the termination of gypsy moth outbreaks in aspen-dominated forests of Michigan in the early 1990s (Lindroth, personal observation). The efficacy of the virus may vary, however, among insects on different aspen trees. Keating et al. (1988, 1990) showed that susceptibility of gypsy moths to LdNPV varied according to host species and attributed the variation to differences in host hydrolyzable tannin contents. In a small-scale, preliminary study (Lindroth and Hwang, 1996a), an inverse correlation

was found between aspen tannin concentrations and pathogenicity of LdNPV to gypsy moth larvae.

The research reported here was conducted to test the hypothesis that variation in aspen chemical composition alters the pathogenicity of LdNPV. We predicted that larval susceptibility to virus would vary in relation to genetic- or resource-based variation in aspen chemistry, particularly phenolics. We conducted two experiments. The first evaluated the effects of clonal variation on foliar chemistry and gypsy moth susceptibility to LdNPV. The second assessed interactive effects of clonal variation and soil nutrient availability on the same variables.

METHODS AND MATERIALS

Experimental Organisms. Quaking aspen clones were originally propagated from root cuttings collected in Dane, Sauk, and Waushara counties, Wisconsin, and Larimer County, Colorado (Hwang and Lindroth, 1997). Small trees were transplanted into 16-liter outdoor pots in May 1993. The soil medium consisted of a 1 : 1 mixture of sand–topsoil (local silt-loam), amended with 3.4 g/liter of slow-release (8–9 months) 18–6–12 (N–P–K) fertilizer. An additional dose (70 g/pot) of fertilizer was added in May 1994. Trees were grown in a common garden on the campus of the University of Wisconsin, Madison.

Gypsy moth larvae were provided by the Otis Methods Development Center (Otis Air National Guard Base, Massachusetts). Prior to use in the viral assays, larvae were reared on gypsy moth wheat germ diet (ICN Biomedicals) in a Percival environmental chamber (15L : 9D; 25°C) in the insect quarantine facility of the Department of Entomology, University of Wisconsin, Madison. Bioassays were conducted with newly molted third instars, which had received no food since molting.

LdNPV (lyophilized "K" 1976) was obtained from Dr. K. Shields (USDA Forest Service, Northeastern Forest Experiment Station, Hamden, Connecticut). The concentrated stock suspension was diluted to bioassay levels with 1% Plyac spreader sticker (Haco, Madison, Wisconsin) in deionized water (pH adjusted to 7).

Clonal Variation Experiment (May 1995). Eight aspen clones were used in this study. Trees were propagated as described previously; no additional fertilizer was provided at the beginning of the 1995 growing season.

LdNPV assays were conducted using 24-well tissue culture plates. For each of five trees per clone, we conducted one replicate assay of 12 insects for a control (1% Plyac solution) and two replicate assays of 24 insects for viral exposure [45,000 polyhedral inclusion bodies (PIB) per insect; total of 300 insects per clone]. Several fully expanded leaves were collected from each experimen-

tal tree. From these, we punched 60 leaf disks (6.5 mm diameter). Leaf disks were placed, one per well, onto 2-μl droplets of water (to reduce desiccation). Each disk was then inoculated with a 1-μl droplet of Plyac solution or LdNPV suspension. After the droplets had dried, single newly molted third instars were placed into the wells. Plates were then covered with a damp paper towel and plastic lid. After feeding for 48 hr, larvae that had consumed entire leaf disks were transferred individually into 35-ml plastic rearing cups containing 10 ml gypsy moth wheat germ diet. Larvae were maintained at room temperature and mortality was recorded after 15 days.

Additional leaf samples were collected from each tree for subsequent chemical analysis (see "Foliar Chemistry" below). Leaves were flash frozen in liquid nitrogen and freeze-dried, then ground (40 mesh) and stored at −20°C.

Clonal Variation × Nutrient Availability Experiment (May 1997). Five aspen clones and two soil nutrient levels were used in this study, conducted as part of a larger study addressing the effects of gene × environment interactions on aspen chemistry. These trees were second "generation" progeny, clonally derived from trees propagated from field-collected root cuttings. Trees were transplanted to 16-liter pots in shadehouses in May 1996. The soil medium consisted of a 3 : 2 mixture of sand-topsoil (local silt-loam). The low nutrient treatment received no fertilizer, whereas the high nutrient treatment was amended with 5.3 g/liter of slow-release fertilizer (8–9 months "Osmocote Plus," 17–6–10 N–P–K, plus micronutrients). An additional dose (85 g/pot) of fertilizer (as previously, but 18–6–12 N–P–K) was added to high nutrient trees in May 1997. Only trees grown in high-light shadehouses (30% shade) were used in this study.

We conducted assays for each of two trees per clone × nutrient combination in each of four shadehouses (blocks). LdNPV assays were conducted as described previously, with the following exceptions: (1) for approximately the last two days of the second stadium, larvae were fed local aspen foliage containing low levels of phenolic glycosides and tannins; (2) for each tree, we conducted one replicate assay for both control (8 insects) and LdNPV (24 insects) treatments; and (3) the LdNPV dose used was 32,000 PIB.

Leaf samples for chemical analysis were collected and processed as described previously.

Foliar Chemistry. Levels of nitrogen, starch, phenolic glycosides, and condensed tannins were measured for foliage collected in both experiments. For nitrogen contents, leaf tissues were first acid-digested (Parkinson and Allen, 1975), then nitrogen concentrations were quantified by a micro-Nesslerization technique (Lang, 1958). Glycine *p*-toluene sulfonate (5.665% N) was used as a nitrogen standard. Starch was measured by the method of M. M. Schoeneberger, K. Ludovici, and P. Faulkner (unpublished manuscript). Starch was extracted, enzymatically converted to glucose, and then quantified indirectly via coupled enzyme reactions that reduce NADP to NADPH. Phenolic glycosides (salicortin

and tremulacin) were measured by high-performance thin-layer chromatography (Lindroth et al., 1993), using phenolic glycosides purified from aspen leaves as standards. Condensed tannin levels were quantified by the acid butanol method of Porter et al. (1986), with purified condensed tannins from aspen leaves (Hagerman and Butler, 1978) serving as the standard.

Statistical Analyses. For the clonal variation experiment, data for insect mortality (square-root arcsine transformed) and plant chemistry were analyzed by one-way analysis of variance (ANOVA) (PROC GLM) (SAS Institute, 1988). Means comparisons were made by the Student-Newman-Keuls' (SNK) test. For the clonal variation × nutrient availability experiment, data for insect mortality (square-root arcsine transformed) and plant chemistry were analyzed as a randomized complete block design (PROC GLM) (SAS Institute, 1998) (clone $df = 4$, nutrient $df = 1$, clone × nutrient $df = 4$, block $df = 3$). For both studies, multiple regression analyses (PROC REG) (SAS Institute, 1988) were conducted to assess relationships between larval mortality and foliar chemical composition.

RESULTS

Clonal Variation Experiment. Viral pathogenicity varied relatively little among caterpillars fed foliage from different clones (Figure 1). Although statistically significant, mortality rates differed by at most 30%. Variation was nearly as great among clones within a common collection site as it was between clones from different sites. No mortality was observed among control insects (data not shown).

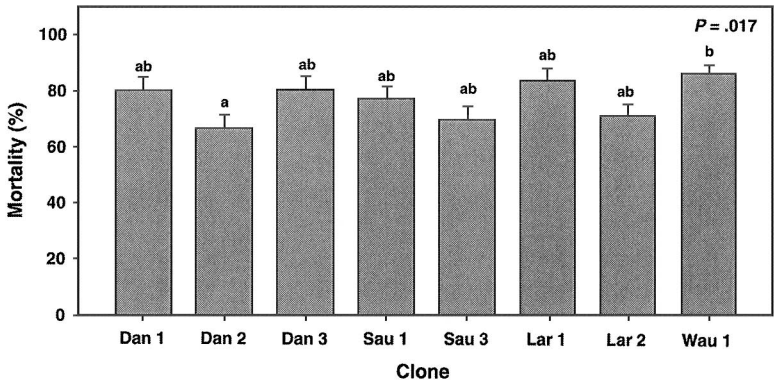

FIG. 1. Clonal variation in gypsy moth susceptibility to LdNPV. Bars bearing different letters are significantly different (SNK test, $\alpha = 0.05$).

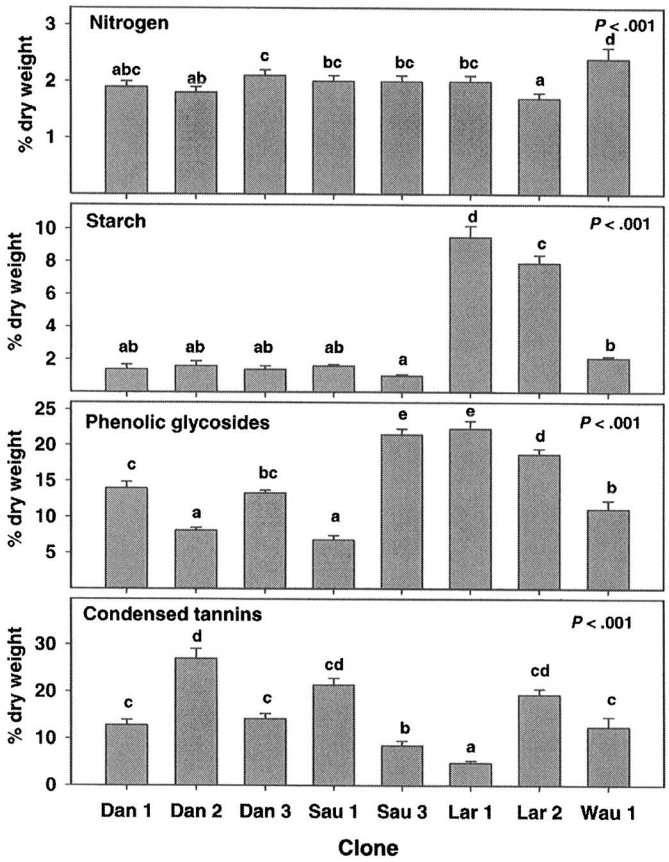

FIG. 2. Clonal variation in aspen phytochemistry. Bars bearing different letters are significantly different (SNK test, $\alpha = 0.05$).

Chemical analyses of aspen foliage revealed marginal (1.4-fold) among-clone variation in nitrogen concentrations (Figure 2). In contrast, starch, phenolic glycoside, and condensed tannin concentrations varied by 9.5-, 3.3-, and 5.6-fold, respectively. Clones from Larimer County had exceptionally high levels of starch. Otherwise, no site-related differences in aspen chemistry were apparent.

Regression analysis showed that viral pathogenicity was most closely, and inversely, related to foliar tannin concentrations. Condensed tannins alone, however, explained only 10% of the variation in NPV mortality; addition of phenolic glycosides and nitrogen to the model improved its explanatory power to 35%.

Clonal Variation × Nutrient Availability Experiment. Viral pathogenicity did not differ among larvae fed foliage from different aspen clones (Figure 3).

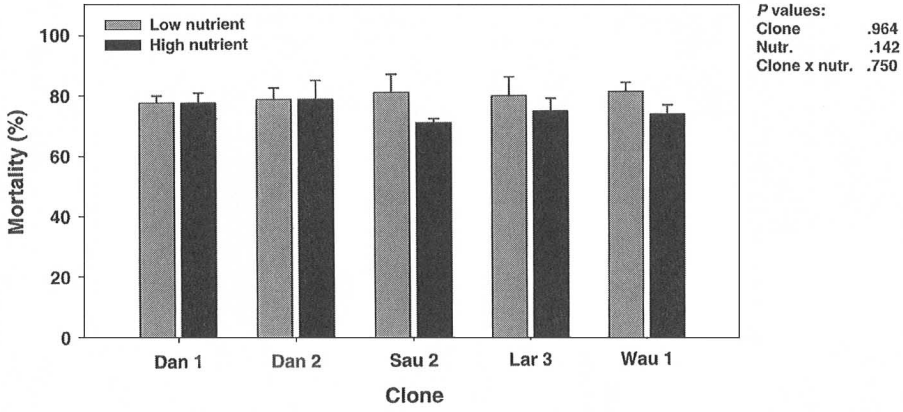

FIG. 3. Clonal and resource-mediated variation in gypsy moth susceptibility to LdNPV.

Mortality tended to decline for insects fed high-nutrient foliage, but nutrient-related differences were of small magnitude and not statistically significant. Clonal genotype did not interact with soil nutrient availability to influence LdNPV pathogenicity. Again, no mortality occurred among control insects.

Aspen chemical composition varied among clones and in relation to soil nutrient availability (Figure 4). Nitrogen contents varied by 24% among clones and increased an average of 31% with nutrient supplementation. Starch concentrations varied by 67% among clones and tended to increase with nutrient availability. Phenolic glycoside levels exhibited 2.6-fold variation among clones and declined an average of 24% in high-nutrient plants. Concentrations of condensed tannins varied by as much as 3.1-fold among clones, and also declined with nutrient supplementation, but not uniformly so among clones.

Regression analysis revealed that larval mortality was not correlated (at $P < 0.05$) with foliar chemical constituents.

DISCUSSION

Susceptibility of gypsy moth larvae to LdNPV was not markedly affected by the chemical composition of aspen foliage. Larval mortality rates remained fairly constant, despite widely varying levels of foliar phenolic glycosides and tannins. In short, we found little support for our hypothesis that chemical variation among aspen clones alters viral pathogenicity and does so in relation to concentrations of phenolic compounds. Condensed tannin concentrations best accounted for the variation in mortality observed, but the relationship was weak.

These results corroborate those of Lindroth et al. (1997), who grew aspen

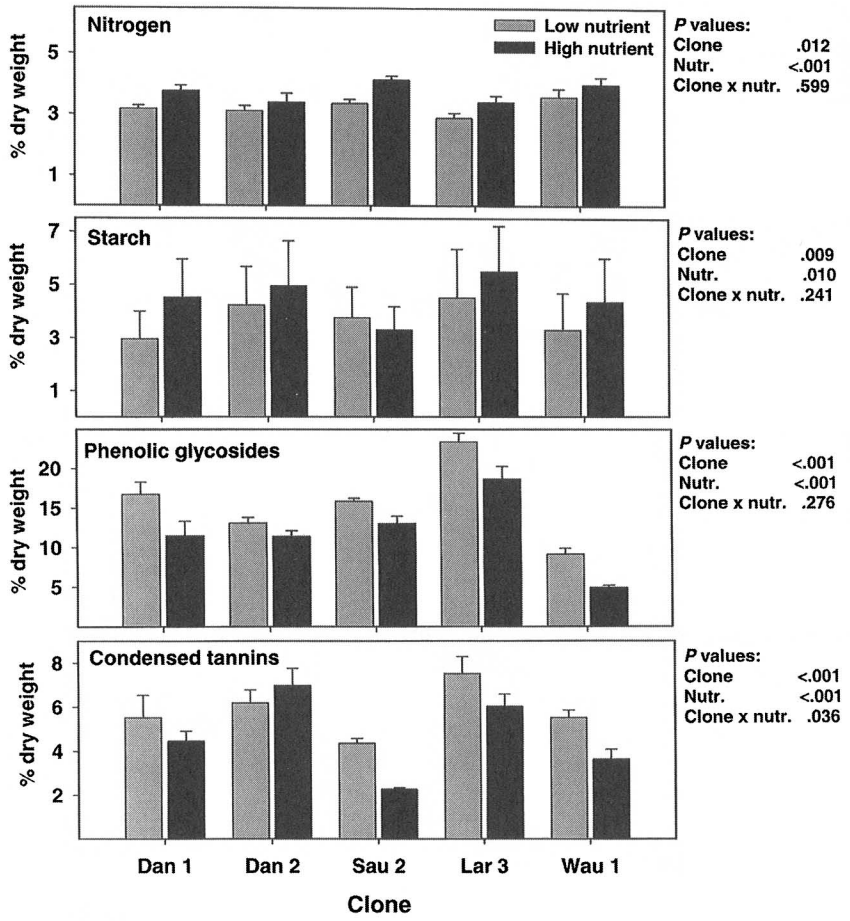

FIG. 4. Clonal and resource-mediated variation in aspen phytochemistry.

in ambient and enriched CO_2 environments and found no effect of host plant on gypsy moth susceptibility to LdNPV. Our results conflict, however, with those of an earlier, preliminary study (in Lindroth and Hwang, 1996a), in which viral pathogenicity varied among four aspen clones and was inversely correlated with foliar tannin levels. The reasons for the discrepancy are unclear, but could involve several factors. For example, the effects of aspen tannins on viral pathogenicity may be greater at lower doses of LdNPV. The earlier study employed a dose of 18,000 PIB, and mortality rates ranged from 17% to 42%. In addition, that study was conducted with mid-season foliage, 3–4 weeks older than that consumed by third instars in the field. The chemical composition of

tannin polymers may shift during that developmental period. Finally, conclusions about host-related differences in viral pathogenicity in the earlier study were at best tentative, as they were based on results from only four plant chemotypes (clones).

Evaluation of our results in the context of related research suggests that the relationship of viral pathogenicity to host-plant chemistry is determined by both qualitative and quantitative characteristics, especially of phenolic substances. Viral pathogenicity is less for insects feeding on oak species than for those feeding on aspen species (Keating et al., 1988). Hydrolyzable tannins, which do not occur in aspen, have been implicated as causal factors in reducing pathogenicity (Keating et al., 1990). For example, induction of gallotannins following defoliation in red oak reduced gypsy moth susceptibility to LdNPV (Hunter and Schultz, 1993). More recently, H. Appel (personal communication) found that for red oak, particular gallotannin size, as well as foliar polyphenoloxidase activity, govern LdNPV inhibition. With respect to condensed tannins, however, both our work and that of Keating et al. (1990) suggest that quantitative variation has relatively little impact on viral pathogenicity. That different classes of phenolics elicit different insect responses to baculoviruses was also demonstrated by Hoover et al. (1998), working with cotton and tomato plants, and noctuid larvae.

That aspen chemistry does not strongly affect LdNPV pathogenicity does not mean, however, that it is generally unimportant in tritrophic interactions. Phenolic glycoside and tannin contents of foliage influence toxicity of the microbial insecticide, *Bacillus thuringiensis kurstaki*, to gypsy moths (Arteel and Lindroth, 1992; Hwang et al., 1995). And research with artificial diets suggests that phenolic glycosides deleteriously affect the gypsy moth parasitoid, *Cotesia melanoscela* (Roth et al., 1997). How variation in aspen chemical composition may influence the susceptibility of aspen-feeding insects to predators and parasitoids in the field, however, remains unknown.

Quantitative variation in the phenolic chemistry (phenolic glycosides and condensed tannins) of trees in our two experiments spanned the range of that exhibited by naturally growing aspen in the Great Lakes area (Hemming and Lindroth, 1995; Lindroth and Hwang, 1996b). Thus, we conclude that clonal or resource-mediated variation in aspen chemistry is unlikely to significantly affect gypsy moth susceptibility to LdNPV in the field. Differential defoliation of aspen is likely due to the direct, bottom-up effects of host chemistry, rather than to the indirect, top-down effects of this pathogen of singular importance to gypsy moth population dynamics.

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BEHAVIORAL RESPONSE OF FEMALE CODLING MOTHS, *Cydia pomonella*, TO APPLE VOLATILES

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Abstract—Apple volatiles stimulated pheromone release, oviposition, and upwind orientation in female codling moths, *Cydia pomonella*. Green apples increased the percentage of virgin females calling, the duration of female calling, and advanced the onset of egg-laying in gravid females. In a tube olfactometer, both virgin and mated females were more active in the presence of apple volatiles than in clean air. They responded by walking while wing-fanning; mated females showed a stronger attraction response than unmated females.

Key Words—Host-plant volatiles, apple, *Malus domestica*, reproductive behavior, codling moth, *Cydia pomonella*, Tortricidae, Lepidoptera.

INTRODUCTION

Codling moth, *Cydia pomonella* (L.) (Lepidoptera, Tortricidae), is the most serious pest of pome fruit orchards worldwide (Chapman, 1973). The control of codling moth by broad-spectrum insecticides affects the beneficial arthropod fauna and increases the risk for outbreaks of secondary pests, such as phytophagous mites. In addition, codling moth populations in all major growing areas have become resistant to a wide range of insecticides. Few alternatives to insecticide treatments are currently available; classical biological control and the use of sterile males have met variable success (Chapman, 1973; White et al., 1973). Mating disruption by synthetic pheromones is a promising and powerful tool for environmentally safe control of codling moth. The most important disad-

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vantage of this technique, however, is that female behaviors are not affected. The use of mating disruption against codling moth is therefore restricted to low population densities and to isolated orchards, where immigration of gravid females is precluded (Trimble, 1995; Minks, 1996; Witzgall and Arn, 1997).

The understanding of how chemical cues mediate female codling moth behaviors is crucial for the development of new complementary control strategies to male orientation disruption. In the field, host-plant volatiles stimulate mating and oviposition in codling moth females (Wildbolz, 1958; Geier, 1963; Jackson, 1979). Despite the importance of host plant volatiles for codling moth reproductive behaviors, few laboratory studies have been done on the mode of action of host stimuli on female behaviors. This is a first report on the diel periodicity of female calling, oviposition, and upwind attraction response to apple volatiles.

METHODS AND MATERIALS

Insects. Codling moths were reared on a semiartificial diet (Mani et al., 1978). The laboratory population originated from the Swiss Federal Research Station Wädenswil (Switzerland) and was regularly interbred with wild insects from Scania (Sweden). Newly emerged females were collected daily, 2 hr before onset of the scotophase (18L : 6D). They were kept in 33-cm^3 Plexiglas containers at 21 ± 5°C, 65 ± 10% relative humidity, and fed with sucrose solution.

Plant Stimulus. Branches with green leaves and apples were cut from a plot of trees of the variety Ingrid Marie, up to 30 min before experiments. By choosing branches from different trees and different parts within tree crowns, the size and ripeness of apples used for experiments was similar over a period of six weeks.

Calling and Oviposition Bioassay. Tests were done during scotophase at 20 lux, 22°C and 65–75% relative humidity. Before onset of the scotophase, individual females were placed into 250-ml plastic containers. One fresh green apple, 3–4 cm diameter, was placed in each treatment container; containers without apples served as controls.

Two-day-old females were used for calling assays. Female calling behavior was recorded at 3-min intervals. Calling females lift the abdomen and protrude the ovipositor. For the oviposition assay, 3-day-old females were mated during the beginning of the scotophase. After copulation, the males were removed and one green apple was placed close to the wall inside each container. The number of eggs and their locations were recorded over two consecutive days.

Olfactometer Bioassay. Female attraction to branches with small green apples and leaves was studied in 4- × 60-cm olfactometer glass tubes. Charcoal-filtered air was blown through a Y-tube into two plastic cylinders (10 × 25 cm), containing either one apple twig ca. 20-cm long with four to six green apples

and some leaves or blank filter paper. An olfactometer tube was fitted tightly into the bottom of each plastic cylinder, and two females were tested simultaneously with and without plant stimulus. The airspeed inside the glass tubes was 30 cm/sec. Air from the downwind end of the glass tubes was exhausted from the room. Prior to testing, the glass tubes were heated to 400°C and the plastic cylinders were washed with detergent and alcohol.

Tests were done with 2- and 3-day-old virgin and mated females during the last hour of the photophase and during scotophase at 21–23°C, 55–65% relative humidity, and a light intensity of 400 and 2 lux, respectively. Two-day-old females were mated at beginning of the scotophase. Mated females were tested immediately during the third and last hour of the scotophase of the same day or kept for testing on the following day. Some of the virgin females started to call in the holding cages before testing during the first hour of scotophase.

All insects were used only once. One female each was released into the downwind end of the control and treatment tubes, respectively, and their behaviors were observed simultaneously at 10-sec intervals for 5 min. The behavioral response of individual females was assessed by quiescence, walking, wing-fanning, and calling. For the scoring of upwind displacement, the olfactometer tubes were divided into six sections of 10 cm. The females were released in section 1; section 6 was at the upwind end.

Statistics. All data were analyzed with Statgraphics software. The level of significance for differences between two means were set to $P = 0.05$ in the Mann-Whitney U test.

RESULTS

Female Calling. The calling behavior of codling moth females was clearly enhanced by green apple volatiles. Females commenced calling earlier and the overall percentage of calling females was higher, compared to the control (Figure 1A). Precalling time (34.6 min) and intervals between calling bouts (30.6 min) were shorter in the presence of green apples than without plant stimulus (56.7 and 43.8 min, respectively; $P = 0.05$). During the first 2 hr of scotophase, there were significant differences in the mean duration of calling among the females in treatment and in control (Figure 1B). Over the entire scotophase, the mean duration of calling per individual female was longer in the treatment (234.7 min), than in the control (180.0 min, $P < 0.05$). The number of calling bouts during the whole scotophase was similar in treatment (1.8) and control (1.6).

Oviposition. The cumulative number of eggs laid per female in the presence of green apple volatiles is shown in Figure 2. Upon mating, the females did not start to lay eggs until shortly before onset of the following scotophase. In the presence of apple volatiles, oviposition peaked 1–3 hr before onset of scotophase,

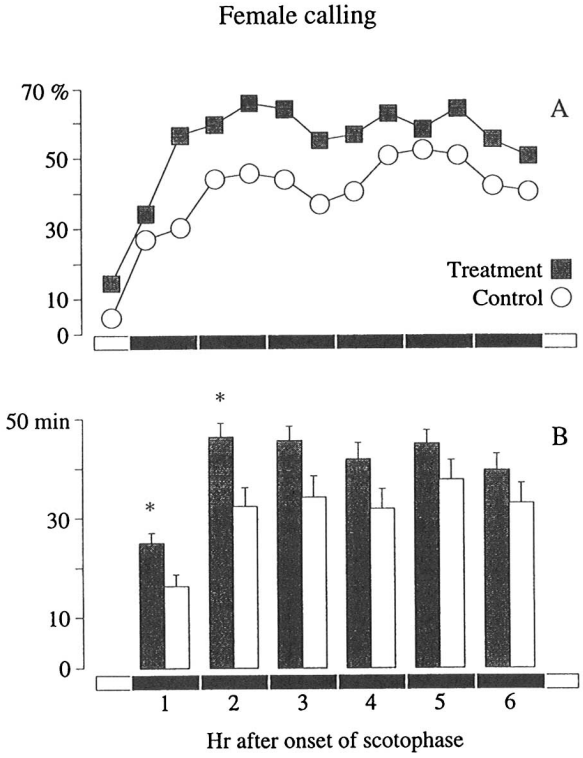

FIG. 1. Calling behavior of codling moth females in the presence of green apples during scotophase (N = 66 and 58 in treatment and control, respectively). (A) Percentage of calling females; (B) duration of calling per female per hr. Asterisks indicate significant differences between treatment and control (Mann-Whitney U test, P = 0.05).

at a rate of 6.6 eggs/female/hr. In the control, the highest oviposition rate of 5.7 eggs/female/hr was observed only after onset of the scotophase. Most of the eggs laid in treatment containers were distributed in the vicinity of apples (79 ± 3% on average), while eggs in control containers were laid randomly. The total number of eggs laid did not differ between treatment and control. During 48 hr, individual females laid 70.0 ± 6.8 eggs in the treatment and 69 ± 8.5 in the control.

Olfactometer Bioassay. The behavioral responses of virgin and mated females to apple volatiles were monitored in a tube olfactometer during the last hour of photophase and during scotophase (Figure 3). Females were quite active in presence of apple volatiles, while females in clean air spent most time in quiescence. Females responded by upwind attraction (displacement) towards the odor

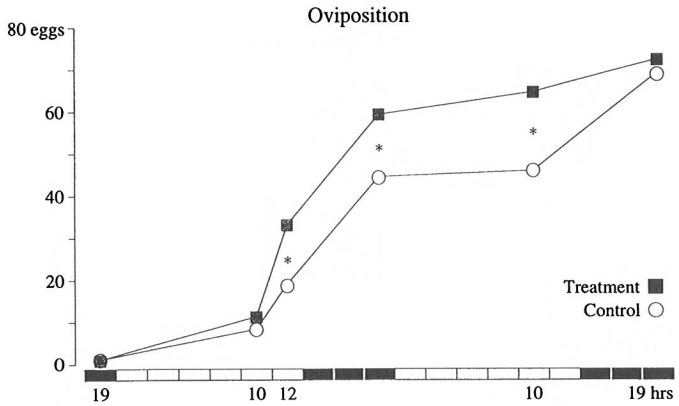

FIG. 2. Cumulative egg numbers laid by codling moth females in the presence of green apples ($N = 15$ in treatment and $N = 16$ in control) over two days. Solid bars: scotophase; asterisks indicate significant differences between treatment and control (Mann-Whitney U test, $P = 0.05$).

source, and they frequently wing-fanned while walking. Mated females showed a stronger attraction response, while virgin females frequently responded by calling, rather than walking and wing-fanning.

Females were active mainly during scotophase, and remained rather quiescent during photophase. However, in presence of apple volatiles, it was observed that virgin females started to call and that mated females responded by wing-fanning even during the last hour of the photophase. There was no difference in the response of females mated during the first hour of scotophase on the same day or of females mated on the day before (Figure 3; M_1 and M_2). During the onset of scotophase, virgin females frequently started to call before being tested (V_c). After these calling females were disturbed when transferred to the olfactometer tube, they quickly resumed calling in the presence of apple odors. Later on during the scotophase, even a few mated females started to call when exposed to apple volatiles.

DISCUSSION

Plant volatiles are widely known to stimulate pheromone production and oviposition in phytophagous insects (Robert, 1986; Hendrikse and Vos-Bünnemeyer, 1987; Raina, 1988; McNeil and Delisle, 1989; Landolt and Philips, 1997). In codling moth, pheromone release and mating behaviors occur during scotophase and are under circadian control; these activities can be modified by environmental conditions, such as light and temperature (Batiste et

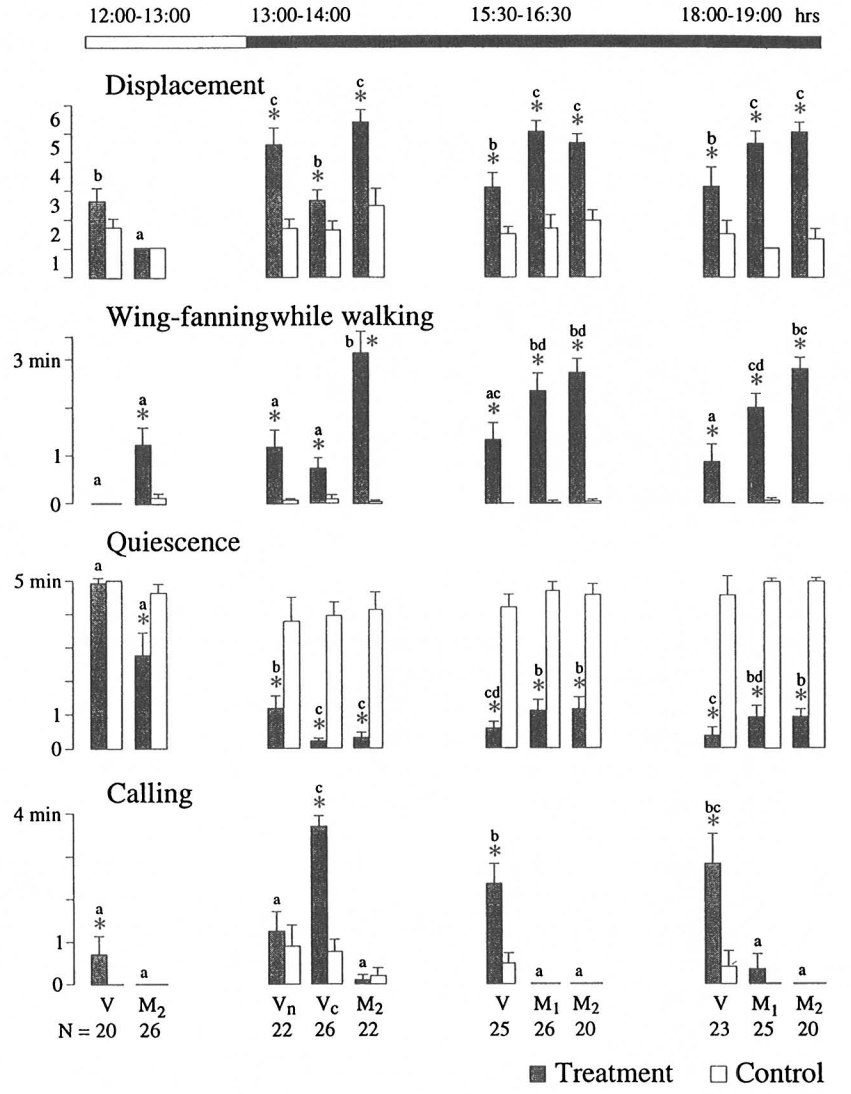

FIG. 3. Behavioral response of female codling moths to twigs with leaves and green apples in a tube olfactometer. Significant differences between treatment and control and between treatments are indicated by asterisks and letters, respectively (Mann-Whitney U test, $P = 0.05$). Solid bar: scotophase. Displacement: position of females in six sections of an olfactometer tube after 5 min. Insects used: 2-day-old virgin females (V); virgin females that did (V_c) or did not call (V_n) before testing; mated females tested on the same day of mating (M_1) and the day after mating (M_2).

al., 1973; Castrovillo and Cardé, 1979). Our results show that the presence of apple volatiles advanced both the onset of pheromone release and oviposition in codling moth females.

Apple odor stimulated egg-laying in codling moth, as suggested by Wearing et al. (1973) and Sutherland et al. (1974). Our data confirm that codling moth had a preoviposition period of about one day in the laboratory (Gehring and Madsen, 1963) and that oviposition did not peak until after beginning of the scotophase in the absence of host-plant stimulus (Weissling and Knight, 1996). However, in the presence of green apples, oviposition peaked 2–3 hr earlier during the last hour of the photophase, which corroborates field observations by Riedl and Loher (1980). There was no difference in the total number of eggs laid in treatment and control. During a short observation period (Sutherland et al., 1974), more eggs may be laid in the presence of apple odors, but the total number of eggs laid over a longer period is similar. Codling moth females contain a constant number of mature eggs during the oviposition period (Deseö, 1972), hence apple odors stimulate egg-laying, possibly by promoting egg maturation (Sutherland et al., 1974).

In the field, most eggs are laid not directly on the fruit, where the concentration of volatile compounds is expected to be highest, but on leaves and twigs nearby (Wildbolz, 1958; Jackson, 1979). It is unclear to what extent close-range ovipositional behaviors are guided by tactile and olfactory stimuli (Curtis et al., 1990; Yokoyama and Miller, 1991). However, the olfactometer bioassay clearly demonstrates that female codling moths are attracted to the oviposition site by volatile compounds from a distance. Females responded to apple volatiles by upwind attraction, and they frequently wing-fanned while walking towards the odor source. Walking accompanied by wing-fanning is otherwise observed in male moths stimulated by female sex pheromone. This characteristic behavioral response can be used in future work to screen compounds identified from apple for their behavioral activity in female codling moths.

(*E*,*E*)-α-Farnesene, an important component of apple volatiles, is known to stimulate oviposition of codling moth females and orientation of larvae (Wearing et al., 1973; Wearing and Hutchins, 1973; Bradley and Suckling, 1995). However, apple fruits and leaves emit a complex blend of compounds (Flath et al., 1969; Yahia et al., 1990; Mattheis et al., 1991; Boevé et al., 1996), and it will be necessary to assay additional compounds alone or in combination with (*E*,*E*)-α-farnesene. Chemical analysis and antennal recordings, together with behavioral studies, will help us to decipher the role of olfactory stimuli in attraction of gravid codling moth females to apple and to develop new strategies for codling moth management.

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CORRECTION FOR DIFFERENCES IN VOLATILITY AMONG OLFACTORY STIMULI AND EFFECT ON EAG RESPONSES OF *Dioryctria abietivorella* TO PLANT VOLATILES

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Abstract—We describe a method using paraffin oil solutions and gas chromatography to measure and correct for differences in volatility among test compounds applied to filter paper and to address the problem of minimizing solvent contributions to EAG responses. To examine the effect of the volatility bias, we evaluated the EAG responses of female fir coneworm, *Dioryctria abietivorella*, to five volatile plant compounds, using a new method to normalize EAG responses to account for the loss of antennal sensitivity that occurs over time. Stimuli were generated either from equimolar (uncorrected) solutions or from corrected solutions that were adjusted to yield equimolar airborne concentrations in the air puffed over antennae. When uncorrected solutions were tested, the two most volatile compounds, (*E*)-2-hexenal and (*E*)-3-hexenyl acetate, elicited significantly larger EAG responses than three terpenes. When corrected concentrations were tested, the ranking of these responses changed: (*E*)-2-hexenal elicited significantly smaller EAGs than (−)-α-pinene, (−)-limonene, and (*E*)-3-hexenyl acetate. On the other hand, there was no effect on the ranking of EAG responses to the two monoterpenes and a sesquiterpene, (−)-*trans*-caryophyllene, relative to each other. Normalization of EAG data did not affect the overall results (i.e., stimulus rankings) but did reduce their variance within preparations. The results show that when compounds with widely different volatilities are compared in olfactory bioassays, the concentrations of test solutions should be adjusted to produce emissions with equimolar airborne concentrations.

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INTRODUCTION

The study of behaviorally relevant olfactory stimuli such as pheromones and host plant volatiles typically involves electrophysiological and behavioral bioassays. Solutions of test compounds are applied to filter paper and then allowed to evaporate for a fixed time in an enclosed space before the volatilized stimuli are delivered via an airstream. A potential problem in establishing the relative effectiveness of stimuli is the sometimes wide differences in volatility among test compounds, which can result in substantial differences in the airborne concentration of the delivered stimuli (Bengtsson et al., 1990; Mayer, 1993). If no correction is made, comparisons among responses to different compounds could lead to false conclusions about receptor sensitivity or behavioral relevance because the magnitude of the responses is potentially biased in favor of the more volatile compounds. Another problem may arise when blends of volatiles are tested because the evaporation of each component from a substrate will depend on its vapor pressure, concentration, and potential interactions between compounds (Barrow, 1988). Hence, the airborne composition of compounds produced by a blend may be different from their proportions in solution.

Although the potential bias caused by volatility differences among host plant stimuli has long been recognized (Guerin and Visser, 1980; Averill et al., 1988; Hartlieb and Rembold, 1996; Shu et al., 1997), few attempts have been made to address this problem (e.g., Jang et al., 1989; Light et al., 1992; Hoskovec et al., 1993). Moreover, the validity of the corrections has not been verified by comparing, for example, responses to corrected and uncorrected stimuli. Wilson et al. (1969) devised a method to quantify evaporation rates and resulting airborne concentrations of volatile stimuli. However, their method was based on a still-air diffusion model for neat compounds evaporating from capillary tubes. It is difficult to apply this method when the stimulus originates from a solution applied to a substrate such as filter paper, as in electroantennogram (EAG) bioassays.

A more practical approach for electrophysiological assays is to assess airborne concentrations of test stimuli by gas chromatographic analysis. Bengtsson et al. (1990) quantified pheromone emissions from treated filter paper held in large syringes to demonstrate that lepidopteran pheromone analogs with different chain lengths have measurable differences in volatility. They developed a regression equation relating volatility and chain length to correct pheromone stimuli for their electrophysiological bioassays. Mayer (1993) has argued that airborne stimuli should be compared on an equimolar rather than on an equal-

weight basis. In his electrophysiological studies, he measured airborne concentrations of a lepidopteran pheromone by capturing the emissions on Porapak Q and quantifying extracts by GC analysis. This method could also be applied to plant volatiles (Hartlieb and Rembold, 1996).

We describe an alternative gas chromatography method to estimate and correct for differences in the airborne concentration of host plant stimuli, including blends. To assess the effect of correcting for volatility differences, we evaluated the EAG responses of female fir coneworm, *Dioryctria abietivorella* (Grote), to five volatile plant compounds with wide differences in volatility by comparing their responses to equimolar (uncorrected) solutions and to corrected solutions that were adjusted, as described below, to yield equimolar airborne concentrations. Because the EAG may be affected by residual solvent from the test solutions, we also addressed the problem of minimizing any solvent contributions to the EAG by examining the relationship over time between the evaporation of the test stimulus and its solvent. Both a volatile and a nonvolatile solvent were evaluated.

Finally, EAG responses are frequently normalized to account for the decline in EAG sensitivity with time and repeated stimulation (Guerin and Visser, 1980; Light et al., 1992; Blackwell et al., 1994) but usually the effect of doing so is not reported. We normalized EAG responses obtained in our experiments using a new approach and show how normalization affects our results.

METHODS AND MATERIALS

Chemicals. Test compounds (Table 1) consisted of two monoterpenes and a sesquiterpene that are potential host-finding cues for insects feeding on conifers (Shu et al., 1997; Brockerhoff, 1998) and two green leaf volatiles, (*E*)-2-hexenal and (*E*)-3-hexenyl acetate that are widely studied as host (Visser, 1986) and nonhost stimuli (Dickens et al., 1992) for other insects. Test compounds and paraffin oil (light mineral oil) were purchased from Aldrich (Milwaukee, Wisconsin), except for (*E*)-3-hexenyl acetate, β-myrcene, and (−)-*trans*-caryophyllene, which were obtained from Bedoukian (Danbury, Connecticut), Sigma (St. Louis, Missouri), and ICN (Mississauga, Ontario), respectively. Purity of the compounds, as determined by GC analysis, was 95–99% except for (+)-Δ-3-carene (94%), *trans*-caryophyllene (92%), and β-myrcene (92%). Impurity was taken into account when solutions were prepared.

Evaporation of α-Pinene vs. Solvent. (−)-α-Pinene (hereafter α-pinene) was chosen as a model host compound to compare the relationship between the evaporation rate of a volatile stimulus relative to that of the solvent in which it is dissolved. Solutions of α-pinene were prepared in two solvents: hexane, a volatile solvent that potentially can contribute to EAG responses, and paraffin

TABLE 1. HEADSPACE CONCENTRATIONS OBTAINED FROM 1 M SOLUTIONS OF TERPENES AND GREEN LEAF VOLATILES IN PARAFFIN OIL AND CALCULATED CONCENTRATIONS REQUIRED TO YIELD A HEADSPACE CONCENTRATION OF 6.7 μM

Chemical	Molecular weight	Headspace concentration (μM) obtained from a 1 M solution[a]	Concentration of solution (mg/ml) yielding headspace concentration of 6.7 μM[b]
(*E*)-2-Hexenal	98.2	106.1	4.5
(*E*)-3-Hexenyl acetate	142.2	46.1	19.7
β-Myrcene	136.2	25.6	35.7
(−)-β-Pinene	136.2	22.1	41.3
(−)-α-Pinene	136.2	21.4	42.7
(−)-Camphene	136.2	20.4	44.9
(−)-Limonene	136.2	17.8	51.3
(+)-Δ-3-Carene	136.2	16.3	56.0
(+)-Camphor	154.3	6.7	152.3
(−)-Bornyl acetate	196.3	4.2	316.9
(−)-*trans*-Caryophyllene	204.4	2.2	605.4

[a]Five microliters of a solution of a test compound in paraffin oil was applied to filter paper, allowed to evaporate for 20 sec, and then sealed in a 2-ml vial. A 5-μl sample of headspace in the vial was obtained 20 sec later and injected into a GC for quantification.

[b]Concentrations were calculated by regression analysis (as in Figure 4). A 1 M (+)-camphor solution, which produces a 6.7 μM headspace concentration, was used as a reference product because it was one of the least volatile monoterpenes among the test compounds.

oil, which is nonvolatile and contributes little to EAG responses. To estimate the headspace concentrations of α-pinene and the solvent, we used the following method: 5 μl of an α-pinene solution in hexane (730 mM = 100 mg/ml) or in paraffin oil (1 M) was applied to filter paper (0.4 × 1.9 cm; Whatman No. 1) and allowed to evaporate for a known period, t_1 (5 sec to 8 min). The filter paper was then placed in a 2-ml glass vial that was sealed with a screw cap fitted with a rubber septum. A 5-μl headspace sample was taken from the vial through the septum with a gas-tight syringe after a fixed period (t_2), which was 30 sec in the case of hexane or 20 sec in the case of paraffin oil,[3] and injected into a capillary GC for quantification. By varying t_1 and repeating these measurements, an approximation of the relationship between the evaporation of the solvent and α-pinene over time could be determined.

Similarly, by applying α-pinene at concentrations ranging from 10 mM to 1 M on the filter paper and keeping t_1 fixed at a convenient value (30 sec for hexane or 20 sec for paraffin oil), the relationship between the applied concentration and the resulting headspace concentration was also determined.

[3]These times were chosen for convenience. The evaporation of solvents and solutes is not affected appreciably by this small difference in the time allowed for evaporation.

Correction of Airborne Concentrations of Different Stimuli. To compare the initial concentration of different stimuli applied to the filter paper with their resulting headspace concentrations, paraffin oil solutions of the five selected plant compounds were tested as described above ($t_1 = t_2 = 20$ sec) to establish by regression analysis the relationship between the concentration of the applied solution (ranging from 10 mM to 3.2 M) and the resulting headspace concentration. The regression equations were then used to derive solute concentrations for each compound to yield the same (equimolar) headspace concentration.

Vapor Pressure of Different Monoterpenes vs. Headspace Concentration. Similar olfactory stimuli with similar molecular weights but with widely different vapor pressures (e.g., monoterpenes) can produce widely different airborne concentrations. To demonstrate this, the vapor pressure of five monoterpenes (camphor, limonene, myrcene, α-pinene, and β-pinene), calculated according to Weast (1970), was compared with their headspace concentrations obtained by applying hexane solutions (730 mM) to filter paper and determining the resulting headspace concentration as described above ($t_1 = t_2 = 30$ sec).

Headspace Composition of Mixtures. The headspace concentrations obtained from a blend of monoterpenes at different concentrations applied in hexane to filter paper were determined as described above for individual compounds ($t_1 = t_2 = 30$ sec). To provide a realistic model, a blend of monoterpenes was selected that matched the proportions emitted by black spruce foliage, *Picea mariana* (Mill.) B.S.P., a host species for *D. abietivorella* (Brockerhoff, 1998).

GC Analysis. Capillary GC analyses were carried out on a Varian 3500 instrument equipped with either a nonpolar HP-1 column [25 m × 0.2 mm ID, film thickness 0.33 μm (Hewlett-Packard, Mississauga, Ontario) with an He flow rate of 1.4 ml/min at 16 psi; injector temperature 250°C; initial column temperature at 40°C for 2 min, then increased at 2.5°C/min to 175°C and held for 10 min; flame ionization detector (FID) temperature was 300°C] or a polar DB-225 column [30 m × 0.25 mm ID, film thickness 0.25 μm (Chromatographic Specialties, Brockville, Ontario); initial column temperature at 50°C for 15 min, then increased at 2°C/min to 190°C and held for 20 min; other conditions as for HP-1]. GC-FID response factors were determined (Raffa and Steffeck, 1988) for all compounds to transform FID counts into amounts.

EAG Responses to Stimuli from Corrected and Uncorrected Solutions. EAGs were recorded from the severed heads of 3-day-old virgin female *D. abietivorella* obtained from a laboratory colony (Shu et al., 1997). Adults were maintained on a 16L : 8D cycle, at 22/20°C and 50% relative humidity. Each preparation was taped to a platform with an antenna exposed to the stimulus source. Ag–AgCl glass capillary electrodes filled with 0.9% NaCl saline were brought into contact with the antennal tip and base and connected via a DC amplifier (Grass P16, Quincy, Massachusetts) to a storage oscilloscope (Tektronix 5111A, Portland, Oregon) to measure the EAG amplitude. Each test compound [(−)-α-pinene, (−)-limonene,

(−)-*trans*-caryophyllene, (*E*)-2-hexenal, and (*E*)-3-hexenyl acetate] was dissolved in paraffin oil at two concentrations: 0.1 M and a compound-specific concentration that resulted in an airborne concentration that was the same (i.e., 6.7 μM) for each stimulus, as calculated by the method in this paper (Table 1). To generate stimuli, 10 μl of test solution were applied evenly to a strip of filter paper (5 × 30 mm, Whatman No. 1) (the amount of test compound applied per square millimeter of filter paper was identical to that used to measure and correct headspace concentrations). After 20 sec, the filter paper was inserted into the center of a disposable glass tube (80 mm long × 5.5 mm ID) connected to a three-way solenoid valve (Cole Parmer, Niles, Illinois) controlling an airstream originating from a tank of compressed medical-purity air. The valve was triggered by a Grass S44 stimulator. Test compounds were allowed to evaporate inside the tube for 20 sec, before a 0.8-sec puff of air (0.5 liters/min) delivered the stimulus. The distance from the tip of the glass tube to the antenna was about 15 mm. A fresh tube was prepared for each stimulus. All stimuli were tested once on the same antenna at 2-min intervals in a randomized order. The first compound tested on each antenna was used as the standard for that run. It was tested again as the last stimulus and the combined initial and final response was used to normalize the EAG data (see below). EAG assays were replicated with 17 female antennae. All bioassays were carried out at 21°C.

Data Analysis. The relationships between headspace concentrations and other parameters were analyzed by fitting linear least squares regression lines or nonlinear models by using Systat's Nonlin function (Systat, 1992). The EAG experiment was designed for a repeated measures factorial ANOVA (Steel and Torrie, 1980). Since mean antennal responses declined almost linearly by about 50% from the initial to the final stimulus, EAGs were normalized to reduce variation not included in the ANOVA factors as follows. On the basis of the responses to the initial and the final stimulus, we determined for each preparation the percentage decline in EAG response for each stimulus and adjusted the EAG data accordingly. The adjusted EAG data were then expressed as a percentage of the highest EAG obtained from each preparation to reduce variability in absolute responses (millivolts) among preparations. ANOVAs were carried out for both raw EAG data and normalized data to examine the effect of normalization. Tukey's multiple comparison test was used to assess the differences between mean EAGs for each data set in trials with the uncorrected and corrected test solutions. All statistical analyses were performed with Systat 5.0 for Windows.

RESULTS

Evaporation of α-Pinene vs. Solvent. Following application of the α-pinene solution in hexane to the filter paper, hexane evaporated completely after about

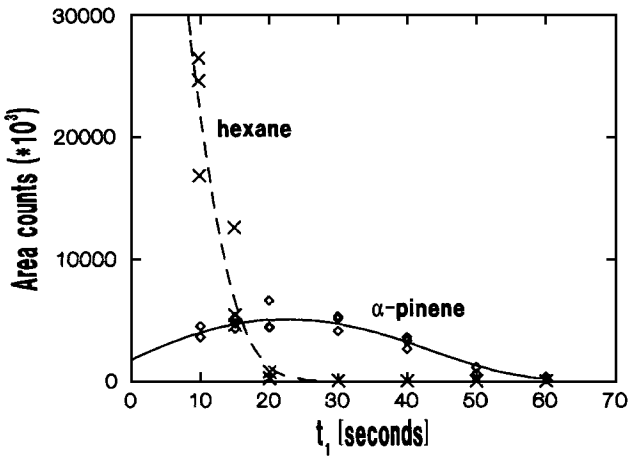

FIG. 1. Relationship between the amount (FID counts) of α-pinene and hexane in the headspace of a closed vial after their evaporation from filter paper for 30 sec relative to the duration (t_1) of their preceding evaporation outside the vial. (Five microliters of a 730 mM solution of α-pinene in hexane was applied to filter paper and placed in a vial after varying lengths of time, t_1; a 5-μl sample of headspace from the closed vial was obtained 30 sec (t_2) later and analyzed by GC.)

30 sec, whereas α-pinene required about 60 sec to evaporate completely (Figure 1). (These times will vary with the quantity of solution applied to the substrate, the nature of the substrate and its area, the solvents, the prevailing temperature, and the experimental protocol). As the logarithm of the concentration of α-pinene in solution increased, the logarithm of the corresponding headspace concentration increased nonlinearly and generally at a much faster rate (Figure 2). For example, when the concentration of α-pinene applied to filter paper increased 10-fold from 73.4 mM to 734 mM, the resulting headspace concentration increased by about 1500-fold (Figure 2).

α-Pinene in a paraffin oil solution (1 M) evaporated at a slower rate than α-pinene in hexane (Figure 3). Even after 8 min, evaporation was incomplete. More importantly, as the logarithm of the solution concentration increased, the logarithm of the resulting headspace concentrations increased in a linear fashion and at the same rate (i.e., slope ≈ 1.0; Figure 4).

Correction of Airborne Concentrations of Different Stimuli. A log-linear relationship with a slope of about 1.0 between the solute and headspace concentrations was also obtained for the other compounds tested (Figure 4) and may be characteristic for paraffin oil solutions in general. Thus, when paraffin oil is used as the solvent, the concentration of test compounds with different volatility can be adjusted to yield similar headspace concentrations. For example, a 1 M solu-

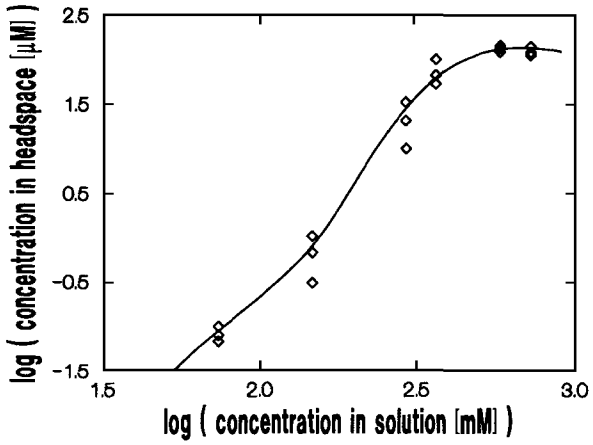

FIG. 2. Relationship between the initial concentration of (−)-α-pinene in a hexane solution applied to filter paper and the resulting concentration in headspace after evaporation for 30 sec in a closed vial. (Experimental procedure as stated in Figure 1 except that t_1 was kept constant at 30 sec).

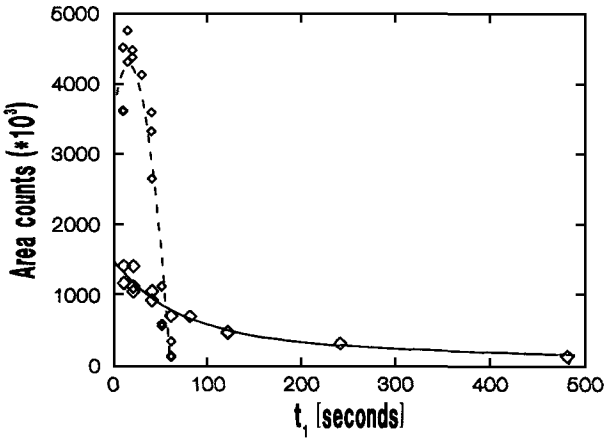

FIG. 3. Relationship between the amount (FID counts) of (−)-α-pinene in the headspace of a closed vial after evaporation from a paraffin oil solution on filter paper for 20 sec in relation to the duration (t_1) of the preceding evaporation outside the vial (solid line). For comparison, the amount of (−)-α-pinene after evaporation from a hexane solution (as in Figure 1) is shown (broken line). (Five microliters of a 1 M α-pinene solution in paraffin oil was applied to the filter paper and placed in a vial after varying lengths of time, t_1; a 5-µl sample of headspace from the vial was obtained 20 sec later and analyzed by GC.)

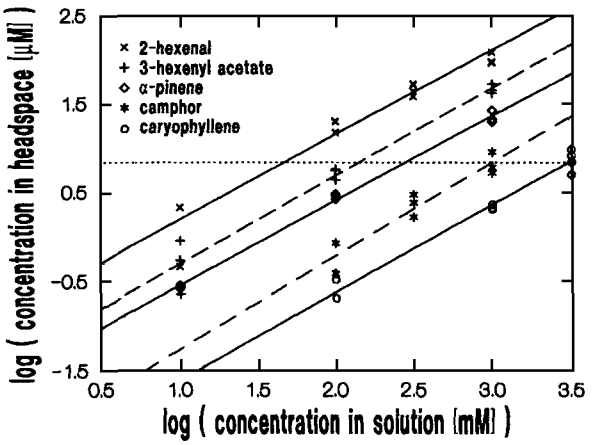

FIG. 4. Relationship between the concentration of five volatile compounds in paraffin oil applied to filter paper and the resulting headspace concentrations after evaporation for 20 sec in a closed vial. All measurements were replicated three times at three concentrations. The horizontal dashed line represents a headspace concentration of 6.7 μM. (Five microliters of solution were applied to filter paper and allowed to evaporate for 20 sec before the filter paper was placed in a vial; the headspace was sampled 20 sec later).

tion of (+)-camphor yielded a headspace concentration of 6.7 μM, whereas only a 0.045 M solution of (*E*)-2-hexenal would be required to yield this headspace concentration (Figure 4). Using the equations of the regression lines for these and other compounds, volatility-corrected solutions, each producing a headspace concentration of 6.7 μM, were prepared for EAG tests (Table 1). The wide differences possible in headspace concentrations produced by equimolar solutions of volatile olfactory stimuli is evident (Figure 4). For example, the headspace concentration produced by the most volatile compound tested, (*E*)-2-hexenal, was 50-fold greater than the headspace concentration produced by an equimolar solution of the least volatile compound tested, (−)-*trans*-caryophyllene.

Vapor Pressure of Different Monoterpenes vs. Headspace Concentration. Differences in headspace (airborne) concentrations were also observed for similar compounds with different vapor pressures. The headspace concentration obtained from closely related monoterpenes dissolved in hexane varied according to their vapor pressure and differed by as much as 10-fold under the same experimental conditions (Figure 5).

Headspace Composition of Mixtures of Compounds. Hexane solutions of monoterpene mixtures (at proportions similar to those emitted by black spruce foliage) produced varying headspace compositions depending on the concentration of the solution (Table 2). With a solution concentration of 100 mg/ml, the

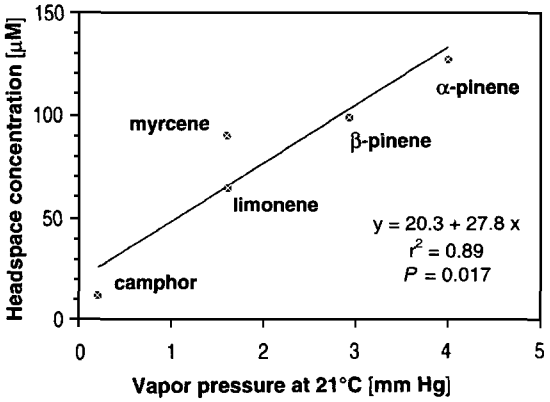

FIG. 5. Relationship between the vapor pressure of some common monoterpenes and their concentration in air after evaporation from filter paper for 30 sec in a closed vial. Vapor pressures were calculated according to Weast (1970). (Experimental procedure as stated in Figure 1 except that t_1 was kept constant at 30 sec).

resulting composition of the headspace was almost identical to that of the solution, but as the concentration of the solution decreased, the proportions of the more volatile compounds also decreased. At 10 mg/ml, only the least volatile compound, bornyl acetate, remained (Table 2); that is, the more volatile compounds had evaporated together with hexane during t_1.

EAG Responses to Stimuli from Corrected and Uncorrected Solutions. Mean EAG (untransformed) responses elicited from female *D. abietivorella* by the test compounds ranged from about 0.3 to 1.8 mV (Fig. 6A). EAGs were significantly influenced by the compounds, the correction for volatility differences, and the antennal preparations (Table 3). Furthermore, the compound × correction interaction was also significant (Table 3), that is, EAGs elicited by corrected and uncorrected stimuli differed relative to each other, and the largest effects were observed for (*E*)-3-hexenyl acetate and (*E*)-2-hexenal relative to the terpenes (Fig. 6A,B). Normalization of EAG data did not greatly affect the overall result (Fig. 6B), but the variance within preparations caused by the decline of EAG responses was reduced (the *F* ratio for preparation dropped from 19.2 to 5.3, Table 3) whereas the *F* ratios for all other ANOVA effects were increased.

When uncorrected (equimolar) solutions were tested, the two most volatile compounds, (*E*)-2-hexenal and (*E*)-3-hexenyl acetate, elicited significantly larger EAG responses than the terpenes (Figure 6B). The ranking of these responses changed when solutions were corrected to compensate for differences in volatility: (*E*)-2-hexenal elicited significantly smaller EAGs than (−)-α-pinene, (−)-limonene, and (*E*)-3-hexenyl acetate (Figure 6B). On the other hand,

TABLE 2. RELATIVE ABUNDANCE OF MONOTERPENES IN HEADSPACE ABOVE MONOTERPENE MIXTURE APPLIED TO FILTER PAPER AT FOUR DIFFERENT CONCENTRATIONS

	Relative abundance of monoterpene percentage (± SE) in				
	Headspace of black spruce foliage[a]	Vapor emitted by filter paper treated with indicated solution concentrations[b]			
		10 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
(−)-α-Pinene	19.1	0.0 (± 0.0)	1.1 (± 0.1)	9.2 (± 0.6)	16.0 (± 0.7)
(+)-Camphene	24.9	0.0 (± 0.0)	2.0 (± 0.3)	15.9 (± 1.0)	24.6 (± 0.7)
(−)-β-Pinene	5.5	0.0 (± 0.0)	1.2 (± 0.1)	4.9 (± 0.2)	6.0 (± 0.0)
β-Myrcene	4.1	0.0 (± 0.0)	2.4 (± 0.2)	4.9 (± 0.1)	4.6 (± 0.1)
(+)-Δ-3-Carene	11.2	0.0 (± 0.0)	4.8 (± 0.3)	11.5 (± 0.4)	10.7 (± 0.3)
(−)-Limonene	3.2	0.0 (± 0.0)	2.3 (± 0.1)	4.2 (± 0.1)	3.4 (± 0.2)
(+)-Bornyl acetate	31.9	100.0 (± 0.0)	86.1 (± 1.2)	49.4 (± 2.5)	34.8 (± 0.8)

[a]Relative abundance of major headspace components (Brockerhoff, 1998) normalized to 100%.
[b]Five microliters of a hexane solution matching the proportions of monoterpenes in the headspace of black spruce foliage was applied to filter paper and allowed to evaporate for 30 sec before it was placed in a vial; the headspace was sampled 30 sec later. GC analyses were replicated twice for each solution.

the correction of concentrations had no effect on the ranking of responses to the monoterpenes and sesquiterpene relative to each other.

DISCUSSION

We applied a GC-based method for estimating airborne concentrations of various dosages of volatile stimuli deposited on filter paper, which is often used in EAG or behavioral bioassays. The results show that dissolving volatile stimuli in a nonvolatile solvent (paraffin oil) enables one to estimate their airborne concentrations and to correct for differences in their volatility because the relationship between the logarithms of the stimulus solution concentration and the resulting headspace concentration is linear, with a slope of about 1.0 (at least for terpenes and green leaf volatiles). This relationship enables olfactory dose–response curves and response threshold levels to be related to airborne concentrations, which is not practical with volatile solvents like hexane because the relationship between the initial solution concentration and the resulting headspace concentration is apparently nonlinear. (Some of this nonlinearity may be an artifact resulting from the condition at $t_2 = 30$ sec when essentially only the neat stimulus remains on the filter paper.) The low volatility of paraffin oil also advantageously avoids the sometimes substantial solvent contributions to the EAGs by volatile solvents like hexane. For example, the mean (± SE) EAG response

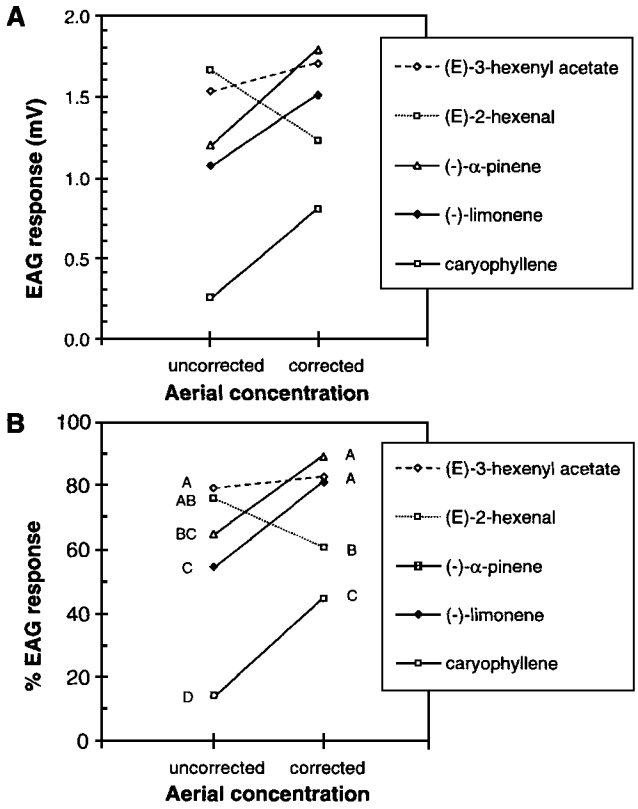

FIG. 6. Mean electroantennogram responses (N = 17) of female *Dioryctria abietivorella* elicited by green leaf volatiles and terpenes each presented at two concentrations: at the same solution concentration (0.1 M) (uncorrected) and the same airborne concentration (corrected). (A) Raw EAG data and (B) normalized data. Different letters beside normalized EAG values indicate significant differences among treatments as determined by Tukey's multiple comparison test.

of virgin female *D. abietivorella* (N = 5) to 10 μl of hexane within 10 sec of its application to filter paper was 1.63 mV (± 0.12), while it was only 0.16 mV (± 0.07) for 10 μl of paraffin oil (Brockerhoff and Grant, unpublished data). However, with volatile solvents, this problem can be avoided by allowing the solution to evaporate for about 25–30 sec to dissipate the hexane, as shown in Figure 1. A disadvantage of paraffin oil is the limited solubility of some test compounds (e.g., borneol) in the solvent.

The significant differences in EAG responses elicited from female *D. abietivorella* by volatility-corrected and uncorrected solutions confirms that cor-

TABLE 3. ANALYSIS OF VARIANCE OF EFFECTS INFLUENCING ELECTROANTENNOGRAM RESPONSES OF *Dioryctria abietivorella* TO TERPENES AND GREEN LEAF VOLATILES

		EAG raw data			Normalized EAGs		
Source	*df*	MS[a]	*F* ratio	*P*	MS	*F* ratio	*P*
Correction[b]	1	6.08	17.7	<0.001	8276.9	41.4	<0.001
Compound	4	7.32	21.4	<0.001	14399.5	72.0	<0.001
Correction × compound	4	1.85	5.4	<0.001	3217.0	16.1	<0.001
Preparation (insect)	16	6.58	19.2	<0.001	1063.6	5.3	<0.001
Error	144	0.34			200.1		

[a]Mean-Square.
[b]Refers to correction for differences in volatility.

rections for differences in volatility of olfactory stimuli are important because response magnitudes are biased towards the most volatile test compounds (Bengtsson et al. 1990; Mayer, 1993). Since molecules are the unit of olfactory detection, airborne compounds should be compared on an equimolar basis (Mayer, 1993). Our results also show that whenever the proportions of a mixture of compounds used in a bioassay of volatiles is important, analyses of the airborne composition of stimuli should also be made and corrected, if necessary.

Our method of correcting airborne concentrations can be laborious if many compounds are to be assayed. However, if a linear relationship with a slope of about 1.0 also exists between the logarithm of the solution concentration and the logarithm of the resulting airborne concentration for compounds representative of chemical classes other than terpenes and green leaf volatiles, then an equation relating the logarithms of the solution concentration and the resulting airborne concentration can be derived from only one concentration for each compound (by assuming a slope of 1.0). Although estimates of the airborne concentrations of test stimuli were based on their headspace concentrations in a closed glass vial rather than in the glass tube (open at one end) holding the EAG stimulus, the relative relationship of airborne concentration in the stimulus delivery system and the headspace concentration should be approximately the same for each compound, particularly when the time from application of the stimulus to the filter paper to its delivery to the antenna is short (20–30 sec).

Finally, when antennal preparations are stimulated repeatedly, sensory fatigue and declining responses usually occur, increasing variation among EAG data. A common method to normalize responses and reduce this variation is the use of a standard stimulus at regular intervals (Guerin and Visser, 1980; Blackwell et al., 1994). However, repeated stimulation with the same compound (i.e., the standard) could increase antennal fatigue to related compounds if the same sensory neurons or receptor sites are stimulated. This could bias the responses

and double the number of stimulations applied to each antenna. Our approach to normalization of EAGs reduces the number of stimulations per antennal preparation and uses all test compounds as the standard, which should reduce potential EAG variability and response bias. However, compounds that elicit a low initial response and a very low or undetectable response to the final stimulus should be avoided as the reference.

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TOLERANCE TO THIARUBRINES FROM *Ambrosia chamissonis* BY THE RARE MOTH *Lasionycta wyatti*

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Abstract—The larvae of a rare noctuid moth, *Lasionycta wyatti*, were found to be associated with the stems and roots of *Ambrosia chamissonis*, a species found along sandy beaches in western North America. High-performance liquid chromatographic (HPLC) analyses of *L. wyatti* larvae and their frass revealed significant amounts of thiarubrines, secondary compounds characteristic of this host plant. Artificial diet studies showed that thiarubrines were well tolerated by these larvae. These same diets, however, were toxic to larvae of *Manduca sexta* and inhibited larval growth of *Spodoptera litura*. Even in the presence of simulated sunlight, artificial diets containing thiarubrines did not affect larvae of *L. wyatti*. However, dietary incorporation of thiophenes, natural UV-activated breakdown products of thiarubrines, was deleterious to larvae of *L. wyatti*.

Key Words—Thiarubrines, thiophenes, allelochemicals, *Ambrosia chamissonis*, *Lasionycta wyatti*, *Manduca sexta*, *Spodoptera litura*.

INTRODUCTION

Members of the Asteraceae alone (Ellis et al., 1995) produce the sulfur-containing polyynes, including the uncommon group based on a 1,2-dithiin skeleton referred to as thiarubrines. "Thia" refers to the sulfur component of the compound and "rubrine" refers to the deep blood-red color of the plant extract. The

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photolabile thiarubrines show both light-mediated and light-independent toxicity against a range of organisms, including bacteria (Towers et al., 1985), fungi (Ellis et al., 1995), and mammals (Truong et al., 1997). Among thiarubrine-producing plants, *Ambrosia chamissonis* produces the most diverse array of thiarubrines (Ellis, 1993).

The toxic thiarubrines are contained within resin canals in the cortex and periderm of stems, leaves, and roots, which run longitudinally along the plant (Ellis, 1993; Ellis et al., 1995). They are believed to confer defense against attack from microorganisms, nematodes, and insects. Guillet et al. (1997) examined the effects of thiarubrine A on neonate larvae of the tobacco hornworm *Manduca sexta* and second-instar larvae of the mosquito *Aedes atropalpus*. Thiarubrine A significantly inhibited the growth of neonate *M. sexta*, with a concentration of 50 μg/g of this compound in the diet resulting in 62% mortality, compared to 5% mortality in the control group (Guillet et al., 1997). The lethal concentration for 50% of the larvae tested (LC_{50}) of thiarubrine A for *A. atropalpus* larvae was 0.09 μg/ml in the dark and 0.18 μg/ml in the presence of near-UV/visible light.

In the present paper we discuss the ability of a rare species of caterpillar to withstand the toxic polyynes found in *A. chamissonis*. The insect was identified as *Lasionycta wyatti* Barn. & Benj. (Lepidoptera: Noctuidae). Until the discovery of *L. wyatti*, *A chamissonis* was believed to be extremely toxic to all microorganisms and insects because of its several toxic sulfur-containing allelochemicals, the thiarubrines and corresponding thiophenes.

Preliminary analyses by HPLC of methanolic extracts of *L. wyatti* larvae collected from the bases of *A. chamissonis* plants indicated the presence of thiarubrines. Based on this finding, the relationship between this insect and plant was investigated further.

One possible way by which *L. wyatti* could acquire thiarubrines is through contact with the chemicals in the sand. Plant leachates, including phototoxins, have been detected in the sand and soil surrounding the bases of several plants. Lam et al. (1989) reported evidence of thiophene-furan acetylene leachates from *Santolina chamaecyparissus* L. (Asteraceae) in sand surrounding *A. chamaecyparissus* plants and α-terthienyl was detected in the soil surrounding *Echinops shaerocephalus* L. (Asteraceae) plants (Lam et al., 1989). Further, Berenbaum and Larson (1988) detected the release of singlet oxygen from *Zanthoxylum americanum* Miller (Rutaceae) (prickly ash) and *Pastinaca sativa* L. (Apiaceae) (wild parsnip) at levels that may cause detrimental effects in insects. Our analyses of the sand samples were negative, however, precluding the possibility that *L. wyatti* larvae acquire the thiarubrines through the integument by their presence in the sand around *A. chamissonis* plants.

A second objective of our study was to determine if *L. wyatti* experienced any of the negative effects of thiarubrines experienced by nonspecialized herbi-

vores. A group of neonate *L. wyatti* larvae was exposed to an artificial diet treated with *A. chamissonis* plant extracts over four days to determine what immediate effect(s), if any, the thiarubrines had on the weight gain of neonate larvae. Along with *L. wyatti*, the same feeding assay was conducted using *Spodoptera litura* and *Manduca sexta* to provide a basis for comparison.

Feeding assays were also conducted in UV/visible light to determine if the activity of thiarubrines would be enhanced with the presence of light. Previous tests on bacteria and viruses showed enhanced activity of thiarubrines in the presence of light against most, but not all microorganisms (Towers et al., 1985; Hudson et al., 1986a,b; Ellis, 1993).

METHODS AND MATERIALS

Field Specimens

Beginning in late March through May of 1996 (with subsequent trips in April and May of 1997), *L. wyatti* larvae, stems from *A. chamissonis* plants, and beach sand were collected at Centennial Beach in Boundary Bay Regional Park, Tsawwassen, British Columbia, Canada. Permits to collect plant materials and insects were obtained from the Greater Vancouver Regional District (GVRD) Parks Department.

After first locating and identifying *A. chamissonis* plants, we carefully dug around the base of randomly selected plants. Sand to a depth of 5–10 cm was brushed aside using either a hand-held trowel, shovel, or simply by hand. Once larvae were uncovered, they were immediately placed in a plastic container and covered with sand.

Stems were collected from plants where *L. wyatti* larvae were found. Stem collections were made from five different *A. chamissonis* plants. Each stem included both above- and below-ground portions. Care was taken to collect stems of similar girth as well as with similar in foliage. Stems were immediately placed in separate brown paper bags that were then placed in a black bag to prevent light exposure.

Four separate samples of sand were collected from the bases of *A. chamissonis* plants. Four Simport plastic specimen containers were wrapped with aluminum foil to prevent exposure of the contents to sunlight. Once larvae were collected from the base of a plant, the lid was removed from the plastic container and sand was scooped into the container at the precise location where larvae were found. The container was filled to the top, immediately capped, and placed into a black bag. Working quickly and physically blocking the sunlight minimized light exposure.

After each collection, the larvae, stems, and sand were brought to the laboratory. Individual larvae were identified by comparing them with previously

identified voucher specimens caught at the same location on previous trips. Voucher specimens are archived at the Canadian National (Insect) Collection (CNC) in Ottawa, Ontario, Canada. A few larvae randomly selected for analyses of thiarubrine content were weighed and immediately frozen to −80°C until extracted. The remaining larvae were used to initiate a laboratory colony. Stem and sand samples were also weighed and immediately frozen to −80°C until extracted.

Laboratory Specimens. In a separate set of analyses, four randomly selected fourth-instar larvae and their frass were taken from the laboratory colony. These larvae were fed a thiarubrine-laced diet for 48 hr, then immediately frozen to −80°C for 24 hr. Frass and the uneaten diet were also frozen to −80°C for 24 hr.

After 24 hr frozen larvae were thawed and dissected. Hemolymph was drawn out by carefully cutting the last proleg with small scissors until no more liquid exuded. A scalpel was used to make a shallow longitudinal incision along the ventral side of each larva. Once the incision was made, the gut contents (excluding mouthparts) were removed with forceps and immediately placed in 3.0 ml CH_2Cl_2. The remaining carcass was placed in 7.0 ml of CH_2Cl_2. All specimens were weighed and stored at 2°C until extracted.

Preparation of Specimens for Analysis. All specimens, with the exception of dissected larvae, were prepared using the following methods. In complete darkness, specimens were thawed, placed in liquid nitrogen, and then crushed in a mortar to a fine powder. A household-type coffee grinder was used in place of mortar and pestle to grind the stem samples. The powders of frass, larvae, and diet were each suspended in 15.0 ml CH_2Cl_2. The finely ground stems were suspended in 50 ml CH_2Cl_2 and sand samples were suspended in 100 ml of CH_2Cl_2. All suspended samples were then extracted for 48 hr at 2°C.

After 48 hr, the solids from all specimens, including the dissected larvae, were removed by vacuum filtration and discarded. The remaining extracts were dried with $MgSO_4$ and concentrated in vacuo. The resulting oily resin was resuspended in 1.0 ml of HPLC-grade CH_3CN. Prior to HPLC analysis, extracts were filtered through a 0.45-μm filter (Gelman) to remove particulates.

HPLC and Spectrophotometric Analyses. Extracts were analyzed by HPLC methods adapted from Page et al. (1997). Water used for HPLC analyses were Milli-Q plus (Millipore), and all other reagents were HPLC grade (Fisher). The HPLC system consisted of a Waters 600 pump controller, a 996 photodiode array detector, and a 717 plus autosampler with separation performed on a C-18 Waters Novapak 3.9 × 150 mm (4 μm) column with a Novapak precolumn. The liquid phase consisted of the following step-gradient water–CH_3CN elution system: 55 : 45 (preinjection); 30 : 70 (0 min); 10 : 90 (10 min); 0 : 100 (15 min); 55 : 45 (32 min). Injection volume was 20 μl with a flow rate of 1 ml/min. UV/visible detection range was set at 200–600 nm with specific detection set at 340 nm.

Spectrophotometric analyses were conducted using a Philips PU 8720 UV/Visible Scanning Spectrophotometer set at a wavelength of 490 nm. All samples were compared against a blank of HPLC-grade CH_3CN. The limit of detection of thiarubrines by the UV spectrophotometer (~5 ppb) was determined with serial dilutions of a known concentration of crude plant extracts. UV spectrophotometric analyses were run on these dilutions until a zero absorbance reading at 490 nm was achieved.

Preparation of Crude Extract from Roots. After 48 hr, the contents of a 4-liter flask containing 117.6 g roots and 2.0 liters CH_2Cl_2 were vacuum filtered through Whatman No. 1 filter paper. The desired extract was obtained by evaporating the CH_2Cl_2 solvent in vacuo at 30°C from the liquid filtrate. The residue was then resuspended in 850 ml of CH_3OH and stored at 2°C.

Conversion of Thiarubrines to Thiophenes. Four hundred millimeters of the stock solution were placed in a stoppered flask and exposed to UV light at an intensity of 6.0 W/m^2, provided by four Sylvania black-light bulbs, for 24 hr at 22°C. After 24 hr, any solids that formed at the bottom of the flask were removed by vacuum filtration.

Preparation of Artificial Diet Containing Crude Thiarubrine Extract. Preparation of thiarubrine-laced and thiophene-laced artificial diets followed the method of Bomford and Isman (1996) with the addition of crude thiarubrines and/or thiophenes extracted from plant materials to produce a final dietary concentration of 150 μg/g fresh weight.

Insects. Field-collected larvae were reared to adults on artificial diet (No. 9795, BioServ Inc., Frenchtown, New Jersey) at 25°C in constant darkness. Identification was made by Mr. J. Troubridge (Agriculture and Agri-Food Canada, Agassiz) and confirmed by Dr. D. LaFontaine (Agriculture and Agri-Food Canada, Ottawa). Altogether, three generations were reared in the laboratory in this manner. Complete details on rearing will be published elsewhere.

The other species used in bioassays were the Asian armyworm, *Spodoptera litura* Hubn. (Lepidoptera: Noctuidae), obtained from a continuous culture in the Department of Plant Science at the University of British Columbia, and the tobacco hornworm, *Manduca sexta* L. (Lepidoptera: Sphingidae), purchased from Carolina Biological Supply Company, Burlington, North Carolina.

Bioassay with Dark Treatment. Two-day-old larvae of each species were collected after hatching and individually weighed (initial weight). Each larva was then placed into a cell of an insect bioassay tray with a 1.0-g food cube. Half the larvae (30–40 individuals, depending on the species) were given food cubes treated with thiarubrines (150 μg/g) and the other half were given untreated food cubes. Food cubes and insect assay trays were replaced with fresh food cubes and clean trays after 48 hr. The larvae remained in these cells with their food cubes for a total of four days in total darkness at 25°C. After four days, each larva was weighed again. In a separate experiment utilizing 4-day-old *L. wyatti*

larvae alone (N = 40), the same methods were used replacing the thiarubrine treated diets with thiophene treated diets.

Bioassays with UV and Visible Light Treatment. Four separate groups of 20 two-day-old *L. wyatti* larvae were used in a two-factor bioassay. Each individual was weighed (initial weight) prior to being placed into a cell in an insect bioassay tray with a 1.0-g food cube. Two groups of 20 larvae were given food cubes treated with thiarubrines (150 μg/g) and the other two groups of 20 larvae were given untreated food cubes. These groups were then placed in total darkness for 48 hr at 25°C. After 48 hr, all larvae were removed from their individual cells and placed into 15-mm × 60-mm Petri plates without any food for 4 hr. During the 4 hr, one group of 20 larvae fed treated diet and one group of larvae fed untreated diet were exposed to UV and visible light at an intensity of 6.3 W/m^2 supplied by two Sylvania cool white bulbs and two General Electric black-light bulbs. In all, 40 individual larvae were exposed to this light treatment. The remaining two groups of 20 larvae were also removed from their cells and placed into 15-mm × 60-mm Petri dishes without food for 4 hr in complete darkness. All four groups were kept at 25°C.

At the end of 4 hr, all larvae were returned to fresh trays and given fresh food cubes. Groups given treated diet were again given treated food cubes at this time. All larvae were then placed in total darkness at 25°C for a further four days. Larvae were transferred to clean trays and provided fresh food cubes every 48 hr. On the fourth day after light treatment, all 80 individuals were weighed again.

RESULTS

Analyses of Plants, Sand, and Field-Collected L. wyatti *Larvae.* Spectrophotometric analyses of sand samples collected from the bases of *A. chamissonis* plants indicated that concentrations of thiarubrines are <5 ppb, the limit of detection, and more than 30,000-fold less than the average concentration in *A. chamissonis* subterranean stems. This precludes the possibility of *L. wyatti* larvae acquiring thiarubrines through direct contact and absorption through the integument.

HPLC analyses of wild *L. wyatti* larvae collected from the same locations around the bases of the plants, however, contained an average of 4.24 μg/g thiarubrines, and frass from these larvae contained an average of 36.8 μg/g of thiarubrines (Table 1). The only means by which wild larvae could have acquired these quantities is through ingestion of plant material. Furthermore, significant subterranean feeding damage was observed from various *A. chamissonis* plants where *L. wyatti* were collected.

The concentration of thiarubrines found in the stems was 147.6 ± 110.2 μg/g dry weight (Table 1). This amount formed the basis for the concentration of

TABLE 1. UV SPECTROPHOTOMETRIC AND HPLC ANALYSES OF SAND, *A. chamissonis* STEMS, WILD *L. wyatti* LARVAE AND THEIR FRASS[a]

Specimen	Concentration (μg/g dry wt)	
	Spectrophotometric analyses	HPLC analyses
Sand	<0.005	nt
Stems	nt	147.6 ± 110.2
Larvae	nt	4.2
Frass	nt	36.8 ± 15.5

[a]Means ± standard deviation; nt = not tested. For sand, stems, and frass, $N = 5$; for larvae, extract was prepared from four larvae pooled.

thiarubrines in the artificial diets. The concentration of total thiarubrines incorporated into the diet was higher than those used in previous tests with other insects (Ellis et al., 1995; Guillet et al., 1997). Moreover, previous tests with insects only used pure thiarubrine A (Ellis et al., 1995; Aucoin et al., 1995; Guillet et al., 1997). In this study, crude extracts were used instead of pure thiarubrines to mimic as closely as possible the true conditions *L. wyatti* larvae are exposed to in nature including the diversity of thiarubrines found in the *A. chamissonis* plants. HPLC analyses of wild larvae and their frass indicated a complex pattern of thiarubrines and thiophenes.

Tissue Distribution of Thiarubrines in Lasionycta wyatti. A 1.0- to 2.0-g cube of artificial diet laced with thiarubrines was given to larvae for 48 hr to determine the fate of the toxins. The highest concentration of recovered thiarubrines appeared in the frass, and relatively little was recovered in the gut contents and integument (Table 2).

TABLE 2. RECOVERY OF THIARUBRINES FROM FRASS, GUT CONTENTS, AND CARCASS OF DISSECTED *Lasionycta wyatti* LARVAE 48 HOURS AFTER ORAL ADMINISTRATION ($N = 4$ FOR EEACH TREATMENT)

Specimen	Recovery[a]	
	μg/g	%
Frass	14.6 ± 4.7	69.7
Gut	6.1 ± 3.7	28.1
Carcass	0.5 ± 0.2	2.2
		% Uneaten
Uneaten diet	36.1 ± 11.2	52.2

[a]Recovery based on estimated quantity of total thiarubrines consumed from diet.

The relatively low levels of thiarubrines found in the gut contents of laboratory reared larvae (Table 2) and whole wild larvae (Table 1) suggest that thiarubrine levels in the insect are kept at very low levels despite the high levels found in plant tissues. Ivie et al. (1983) reported that *Papilio polyxenes* contained only 0.7% of the administered amount of the furanocoumarin, xanthotoxin, in its body (Ivie et al., 1983). Similarly, wild-caught *L. wyatti* larvae contained only 2.9% of the amount of thiarubrines found in stems (Table 1).

The analysis of wild larvae uncovered an unidentified peak that was not seen in chromatograms of stem or root extracts. This peak may represent a metabolic breakdown product of a thiarubrine; its UV spectrum is not consistent with either a thiarubrine or a corresponding thiophene. It is not known at this time if the conversion of the thiarubrines to their corresponding thiophenes can be achieved enzymatically or if the conversion of thiarubrines can result in products other than their corresponding thiophenes. Identification of this unknown compound may help to answer these and other questions.

Comparative Biological Effects of Thiarubrines on Larval Weight Gain of L. wyatti, S. litura, *and* M. sexta *Larvae.* The incorporation of crude thiarubrine extracts from *A. chamissonis* plants into an artificial diet significantly ($P < 0.01$) reduced weight of 4-day-old *S. litura* and *M. sexta* larvae (Table 3) in complete darkness, compared to cohorts fed untreated diets. Larval growth of *S. litura* fed the thiarubrine-laced diet was inhibited by 25% with no mortality. *M. sexta*, however, experienced greater than 50% mortality within four days of the exposure (Table 3). In contrast, thiarubrine-laced artificial diet did not cause any mortality or significant reduction in larval weight gain in *L. wyatti* ($P > 0.05$; Table 3).

The light-dependent effects of thiarubrines on *L. wyatti* larval weight gain were tested to determine if light enhances the effects of thiarubrines in an artificial diet. Results showed that exposure to UV/visible light had no significant

TABLE 3. AVERAGE WEIGHT GAINED BY NEONATE LARVAE EXPOSED TO ARTIFICIAL DIET TREATED WITH AND WITHOUT THIARUBRINES OVER 4 DAYS IN COMPLETE DARKNESS[a]

	Larval weight gain (mg)			
Species	Treated diet (150 μg/g)	Untreated diet	*P*	Mortality (%)
L. wyatti	11.6 (± 0.8)	10.3 (± 0.2)	>0.05	0.0
S. litura	19.9 (± 0.4)	26.3 (± 0.3)	<0.01	0.0
M. sexta	0.8 (± 0.0[b])	24.0 (± 0.1)	<0.01	57.5

[a]Means (± standard deviation). For *L. wyatti*, $N = 30$, 15 (two replicates); for *S. litura*, $N = 40$, 20 (two replicates); for *M. sexta*, $N = 40$.
[b]Standard deviation = 7.3×10^{-7}.

TABLE 4. TWO-WAY ANOVA OF WEIGHT GAINED BY 4-DAY-OLD *Lasionycta wyatti* LARVAE EXPOSED TO ARTIFICIAL DIET WITH AND WITHOUT THIARUBRINES IN BOTH DARKNESS AND UV/VISIBLE LIGHT[a]

	Two-way ANOVA			
Source	*df*	MS	*F*	*P*
Diet	1	13.800	3.786	>0.05
Light	1	1.836	0.501	>0.05
Days[b]	1	250.627	NT	
D × L	1	0.000	0.000	>0.05
Error	3	3.666		

[a]NT = not testable. Treatments are fixed.
[b]Replicates conducted on different days.

effect ($P > 0.05$) on larval weight gain of *L. wyatti* larvae (Table 4). At the onset of feeding trials, larvae readily ate artificial diets. There was no visible difference in the amounts of diet ingested by larvae fed untreated diet from those fed the treated diet. A two-way analysis of variance (ANOVA) showed no interaction between the presence of light and thiarubrines. Therefore, the combination of dietary thiarubrines and the presence of near UV/visible light (Table 4) did not affect *L. wyatti* larvae. The weight gained by *L. wyatti* larvae in the feeding trials, however, differed between the replicates.

In the first feeding trial, larvae fed the treated diet fared better than their cohorts fed an untreated diet, irrespective of the presence of UV/visible light. This result was not seen in the second feeding trial. There was no significant difference in the weight gain of larvae fed the treated diet compared to those fed the untreated diet. However, we observed visible differences in groups fed treated versus untreated diet over the entire life cycle. Those larvae fed the treated diets fared better overall. Larval molting patterns were more uniform and the length of time between each molt was shorter.

Effect of Thiophenes in Artificial Diet on Larval Weight Gain of Lasionycta wyatti *Larvae.* A crude extract of thiarubrines, photoconverted to their corresponding thiophenes with the use of UV light, was incorporated into an artificial diet and fed to 4-day-old *L. wyatti* larvae. Results showed that the thiophenes significantly affected the larval weight gain of *L. wyatti* larvae. Over two replicates, the average weight gained by 4-day-old *L. wyatti* larvae exposed to a 150 μg/g thiophene-laced diet reared in dim light was 8.6 ± 0.2 mg while the average weight gained by cohorts fed an untreated diet was 15.0 ± 1.2 mg ($P < 0.001$).

DISCUSSION

L. wyatti fed an artificial diet laced with thiarubrines over their entire lives did not experience any problems in their development. There was some evidence that larvae fed an artificial diet with thiarubrines may have actually incurred some benefit over conspecifics fed untreated diet (data not shown). Larvae fed the treated diet molted more consistently than those fed an untreated diet, and the days between each molt was shorter than those fed the untreated diets. This observation lends further support to the hypothesis that *L. wyatti* larvae specialize on *A. chamissonis* and are tolerant of thiarubrines.

Furthermore, tests conducted in dim light showed that the larval weight gain of neonate *L. wyatti* larvae was not significantly affected while larval weight gains of *S. litura* and *M. sexta* were (Table 3). Thiarubrines show a complex spectrum of toxicity against microorganisms that includes light-independent (dark) toxicity and visible- and UV-mediated phototoxicity (Constabel and Towers, 1989). The results from tests against *S. litura* and *M. sexta*, as well as other insects (Ellis et al., 1995; Aucoin et al., 1995; Guillet et al., 1997) suggest that thiarubrines are not just phototoxic to insects, but are directly toxic as no light was necessary to produce negative effects.

S. litura larvae exposed to the treated diets did not seem deterred by the presence of toxins in the diet as there was no major difference in the accumulation of frass between larvae fed control diets and those fed the treated diets. However, the growth of neonate *S. litura* larvae was inhibited (Table 3). This suggests that the effects of thiarubrines in *S. litura* are physiological and not behavioral, as they were with the cockroach *Blatta orientalis* (Ellis et al., 1995) and the European corn borer *Ostrinia nubilalis* (Aucoin et al., 1995). *B. orientalis* simply avoided all contact with commercial dog food laced with 10 μg/g thiarubrines in a dose-dependent manner.

M. sexta, however, was not deterred by the thiarubrines, even though it is highly susceptible to α-terthienyl and other phototoxins (Downum et al., 1984; Champagne et al., 1986; Iyengar et al., 1987; Fields et al., 1990; Aucoin et al., 1995; Guillet et al., 1997). In our study as well as a previous study conducted by Aucoin et al. (1995), *M. sexta* appeared unable to detect the toxins, feeding readily on treated diets. Aucoin et al. (1995) determined the index of consumption for *M. sexta* larvae feeding on diet containing thiarubrine A. They found that larvae did not change their consumption rate as the dietary thiarubrine concentration increased, and they became intoxicated. In the present study, over 50% of the *M. sexta* larvae given an artificial diet treated with 150 μg/g of thiarubrine extract died within two to three days of exposure. In spite of this, *L. wyatti* was able to tolerate dietary thiarubrines.

To determine if the activity of thiarubrines against *L. wyatti* was enhanced by light, as was the case against bacteria (Towers et al., 1985; Ellis, 1993), or light-

dependent, as shown for viruses (Hudson et al., 1986a,b), neonate *L. wyatti* larvae were subjected to UV/visible light in combination with a thiarubrine-laced diet over four days. The two-way analysis of variance (ANOVA) showed that the individual factors "diet" (presence or absence of thiarubrines) or "light" (presence or absence of UV/visible light) did not affect the weight gain of *L. wyatti* larvae (Table 4), nor did the combination of these two factors (Table 4). It appears from our data that *L. wyatti* are tolerant of the toxic effects of the thiarubrines under the conditions tested. The ability to escape thiarubrine toxicity may be a function of this species' ability to eliminate the toxins rapidly from its tissues.

Although the *F* statistic is not testable because the variables are fixed, the data from the first feeding trial suggested that larvae fed the treated diet gained more weight over four days than their cohorts fed the untreated diet, regardless of the presence of light. These experimental group larvae fared better than the control group. However, in the second trial, this result was not seen. There was no significant difference in the weight gain of larvae fed the treated diets compared to those fed the untreated diet. Since visual observations also suggest that larvae on the treated diets had a more uniform molting pattern than cohorts fed the untreated diet, further experiments could show that *L. wyatti* do in fact fare better and may even require a thiarubrine-rich diet. This result is an interesting one and warrants further replicate trials. Unfortunately, more replicate trials could not be performed because our laboratory colony of *L. wyatti* was lost after three generations. Additional replicates would have required further field collection of larvae and therefore an additional year to complete.

The enhancement of thiarubrine activity by UV light reported in past experiments (Towers et al., 1985; Hudson et al., 1986a,b; Ellis, 1993; Guillet et al., 1997) is likely due to the photoconversion of thiarubrines to their corresponding thiophenes followed by UV-mediated photosensitization of the thiophenes formed in this manner. However, it is also apparent that the photoconversion process itself, which gives a series of short-lived intermediates (Block et al., 1996), may also contribute to the toxicity of thiarubrines under UV and visible light (Constabel, 1988).

Because of this ambiguity, separate feeding assays were conducted with thiophenes. An *A. chamissonis* extract, in which all the thiarubrines had been converted to their corresponding thiophenes, was incorporated into an artificial diet and fed to 4-day-old *L. wyatti* larvae. *L. wyatti*'s ability to eliminate the thiarubrines from its tissues rapidly may prevent the accumulation of thiarubrines and thereby block the in vivo photoconversion of the thiarubrines to their corresponding thiophenes. In their natural habitat, *L. wyatti* larvae are found below the surface of the sand, and therefore exposure to thiophenes (activated by UV light) is very unlikely to occur.

The incorporation of thiophenes into an artificial diet revealed that *L. wyatti* larvae were susceptible to them. *L. wyatti* exposed to a thiophene-laced diet gained

significantly less weight than their cohorts fed an untreated diet. This result is noteworthy because thiophenes have not exhibited pronounced light-independent (dark) toxicity against microorganisms or insects in previous bioassays (Towers et al., 1985; Guillet et al., 1997). A possible explanation is that UV treatment of the *A. chamissonis* extract for 24 hr led to the formation of compounds, such as sulfur derivatives formed by the extrusion of sulfur as part of the photoconversion of thiarubrines, that are toxic to *L. wyatti*. That thiophenes were possibly responsible for the reduction in larval weight gain also suggests that should *L. wyatti* consume thiarubrines and then be exposed to UV light, they would be photosensitized. The reason why the light treatment in the factorial feeding assay did not reveal any effects on larval weight gain of *L. wyatti* may be due to the inability of light to penetrate to the insect's digestive tract or other tissues where the thiarubrines occur. It is unlikely that metabolism plays a role, as thiarubrines can be converted to thiophenes by sunlight in less than a minute. The duration of the light exposure treatment in the factorial feeding assay lasted 4 hr at 6.3 W/m^2. Realistically, noctuids like *L. wyatti* would not be exposed to UV light in their natural habitats for more than a few seconds at most, since they display light avoidance behavior. Thus, photosensitization by activated thiophenes is highly unlikely.

The results of the thiarubrine feeding assays conducted in darkness and in UV/visible light strongly support the notion that *L. wyatti* are tolerant of *A. chamissonis* plant extracts rich in thiarubrines as long as the exposure to UV is minimized. Furthermore, wild *L. wyatti* larvae and their frass contained thiarubrines, which suggests that they are feeding on *A. chamissonis* plants in their natural habitat. Moreover, the sites along the west coast of North America where *L. wyatti* have been collected closely resemble the distribution pattern of *A. chamissonis*. Previous host records for this species include beach buckwheat [*Polygonum paronychia* Cham. & Schltdl. (Polygonaceae)], beach verbena [*Abronia latifolia* Eschsch. (Nyctaginaceae)], and seaside tansy [Tanacetum camphoratum Less. (Asteraceae)], common plants not normally characterized as toxic or noxious (Crumb, 1956; Godfrey, 1972). A detailed field study of *L. wyatti* and *A. chamissonis* may reveal that this relationship is an example of a host-plant shift that could culminate in sympatric speciation.

In the present study, *L. wyatti* larvae were found only on this toxic host and not on adjacent plants. The fact that larvae were recovered in consecutive years from the same host plant in the same location strongly suggests a resident population of *L. wyatti* larvae specializing on *A. chamissonis*. The underground lifestyle of this insect coupled with its physiological ability to tolerate thiarubrines (and possibly other toxins in *A. chamissonis*) supports the idea that *L. wyatti* is adapted to this host plant.

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SPECIES RECOGNITION FROM POSTPHARYNGEAL GLAND CONTENTS OF ANTS OF THE *Cataglyphis bicolor* GROUP

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Abstract—The *Cataglyphis bicolor* group of species of desert-dwelling ants, difficult to identify from morphological features alone, can be readily recognized by the contents of their postpharyngeal glands. Analysis by linked gas chromatography–mass spectrometry of glands from colonies identified only by code numbers showed in all samples straight and branched-chain alkanes and linear alkenes. *C. viaticus*, *C. bicolor*, and *C. savignyi*, the three species most difficult to distinguish morphologically, each contained distinctly different patterns of hydrocarbons, as illustrated by cluster analysis. The 16 most abundant hydrocarbons in the whole group of samples were selected and plotted as windrose diagrams. The differences in the windroses have more visual impact than gas chromatograms of the same data. The only case where there was any similarity was that between *C. bicolor* and *C. diehlii*, and even there the resemblance was not close. *C. bombycinus* is a sympatric species but is recognized as not belonging to the *bicolor* group by its different mandibular gland substances. It also was easily distinguished by its postpharyngeal gland contents from the other species.

Key Words—Formicinae, ant, exocrine secretion, hydrocarbons, species specificity, species diagnosis, postpharyngeal gland.

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INTRODUCTION

The work of Wehner (1994) on navigation in desert-dwelling *Cataglyphis* ants kindled our interest in this group and the possible use of semiochemicals to aid in distinguishing the species. There are over 100 described taxa of *Cataglyphis*, almost all living in desert or semidesert habitats, but it is very difficult to identify species from morphological characters alone. Santschi (1929) left a tangle of species and subspecific names for *Cataglyphis* from his years of work in Tunisia. Much of Wehner's work has been carried out on "*Cataglyphis bicolor*," but during the course of the work it became clear that either this species varied in appearance from place to place or it was really a group of species. The work of Agosti (1990) clarified the picture considerably, and our preliminary chemotaxonomic studies showed that identification of exocrine secretions could aid the process (Keegans et al., 1992). We have further shown that the *Cataglyphis bicolor* group shares a mandibular gland secretion that consists essentially of one compound, (*S*)-2-methyl-1-hexanol (Agosti et al., 1996), but as yet of unknown function.

The function of the postpharyngeal gland of ants also remains unknown in spite of various speculations. Some of us have shown that the postpharyngeal glands contain the same mixture of hydrocarbons as is found on the cuticle of at least a number of ant species selected at random (Bagnères and Morgan, 1991). Subsequently Hefetz's group has, through several publications, studied the relation between the cuticular and postpharyngeal hydrocarbons (Hefetz et al., 1996). There is much accumulated evidence indicating that the cuticular hydrocarbons are used in nestmate or species recognition in many insect classes (Singer and Espelie, 1996; Bonavita-Cougourdan et al., 1997; Doi et al., 1997; Heifetz et al., 1997) and it is currently thought that the hydrocarbons of the postpharyngeal gland play some part in this recognition.

In the present study, we have taken a chemotaxonomic approach to see if the postpharyngeal gland substances were helpful in defining species within the *C. bicolor* group. At the same time we have explored the use of diagrammatic representation to make differences and similarities in multicomponent mixtures readily apparent. Cluster analysis shows the distinct separation of species. We find that windrose diagrams are very helpful for this group. They can, at a glance, distinguish species. Windrose diagrams, first used to show the relative strength and frequency of winds at a given locality, are compact and visually striking.

METHODS AND MATERIALS

The ants were collected at sites in Tunisia and Egypt by D. Agosti during 1992 [for location see Wehner et al. (1994)]. The nests used for this study were also used in a study of Dufour glands (Gokçen et al., unpublished). The nests

were brought live to Zürich where the postpharngeal glands were dissected out as described by Morgan (1990) and sealed in glass capillaries for transport to Keele. There they were analyzed by linked gas chromatography–mass spectrometry (GC-MS), avoiding the use of solvent and injecting the glands directly into the gas chromatograph by the method of Morgan and Wadhams (1972). Ten individuals from each of 12 nests were analyzed but only five and seven individuals were available from the two nests identified as **gbom** and **kbom**, respectively.

The GC-MS was performed with a Hewlett-Packard 5890 gas chromatograph linked to a 5970B Mass Selective Detector, a quadrupole mass spectrometer using 70 eV ionization, and set to monitor m/z 35–450. The system was controlled by an HP Series 300 computer with HP 59970C ChemStation software. A fused silica capillary column [12 m × 0.32 m × 0.25 mm film thickness of 5% phenyl–95% methylsiloxane (non-polar) phase; SGE, Milton Keynes, UK] was used for chromatography. A surface-deactivated, fused silica retention gap (1 m × 0.2 mm ID) at the injection end was used to protect the column, with a similar piece of deactivated fused silica capillary (5 m) between the column and the mass spectrometer to ensure the column was operating under a positive pressure of helium, which was used as carrier gas at 1 ml/min. The samples were heated in the injector at 250°C for 3 min before crushing the glass capillary.

The column was initially at 100°C for 2 min, then heated at 8°C/min to 280°C, and finally held at that temperature to give a total run time of 40 min. The injector was operated splitless for the injection and 30 sec after.

Identification of the compounds was made chiefly by comparison of retention time and fragmentation pattern with a series of straight-chain hydrocarbons from C_{10} to C_{30} and with the aid of several mass spectral libraries. The presence and position of methyl branching in hydrocarbons was identified from characteristic mass spectral fragmentation. The positions of double bonds in alkenes were not determined. For quantitative analysis, peak areas for each component in the chromatogram were determined by computer integration, and the percentage of each substance in the gland was calculated. The average percentage composition was calculated for each colony together with the sample standard deviation (SD) for each of the compounds, and then the same was done for each group of colonies identified by the same code.

For cluster analysis the program Systat 6.0 (SPSS Inc., Chicago Illinois, USA) was used. Mean hydrocarbon composition for each species was standardized and the standardized data submitted to hierarchical cluster analysis using Euclidian distances.

The 16 most abundant compounds in all colonies and species were selected from the average values of these compounds for each species, and windrose diagrams were constructed for these 16 as they occurred in each colony. The mean amount of each substance in each colony was plotted as the area of the segment of the circle for Figures 1–3.

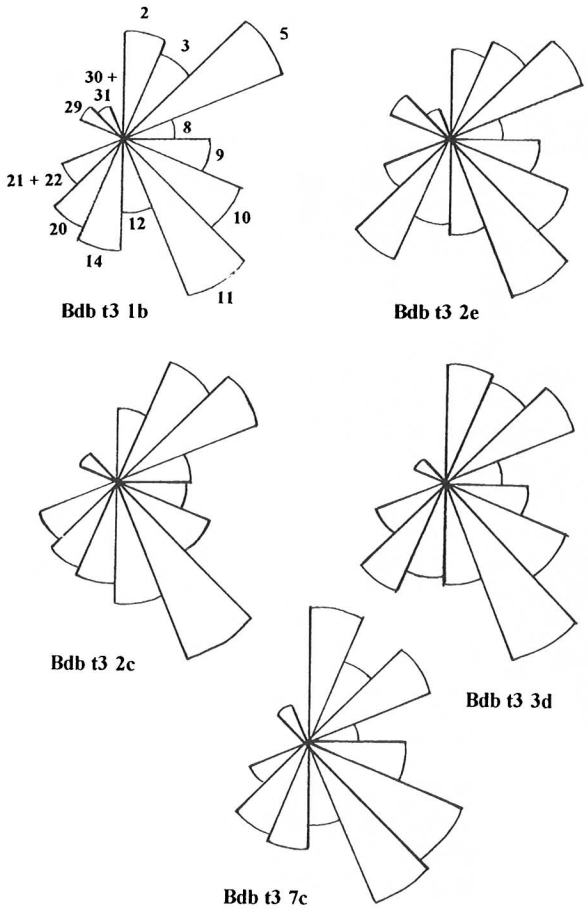

FIG. 1. Windrose diagrams of the mean amounts of the principal hydrocarbon constituents of the postpharyngeal glands of five colonies of ants identified as **bdb** (*Cataglyphis bicolor*). The names of the numbered components are given in bold in Table 1. The designation t3 represents transect 3 of Tunisia, and 1b, 1c, etc., indicate the place of collection and the colony. For locations, see map in Agosti et al. (1995).

RESULTS

The study was carried out blind. Colonies of the *C. bicolor* group were collected at various sites in Tunisia, identified only by a code [e.g., t3 indicates the transect (see Wehner et al., 1994; Agosti et al., 1996); 1–10, the collection point on the transect, and a–d, the colony collected at that point, and three letters

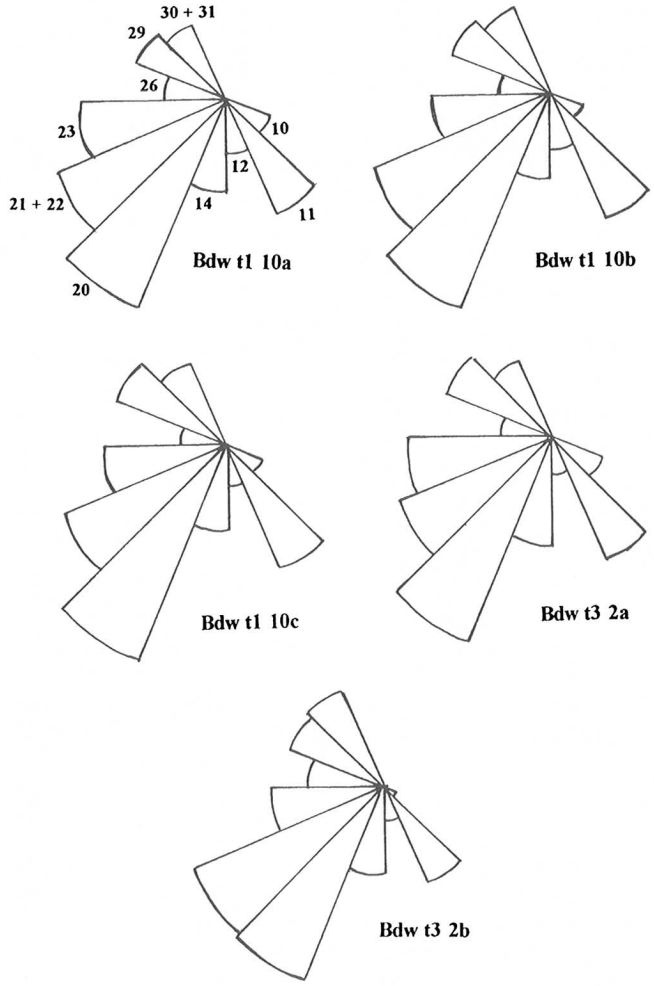

FIG. 2. Windroses for the mean amounts of the principal hydrocarbons in the postpharyngeal glands of five colonies of Tunisian ants identified as **bdw** (*Cataglyphis savignyi*). The location description is as in Figure 1.

which referred to some morphological character, Table 1]. The code names were only united with the new species names (*bicolor*, *diehlii*, *savignyi*, *viaticus*, and *bombycinus*) derived from morphological examination of the male genitalia, after the analyses were finished, the colonies grouped by type and reported. A total of 14 colonies were included in the study.

There were 33 hydrocarbon peaks recognized in the study, not all of them

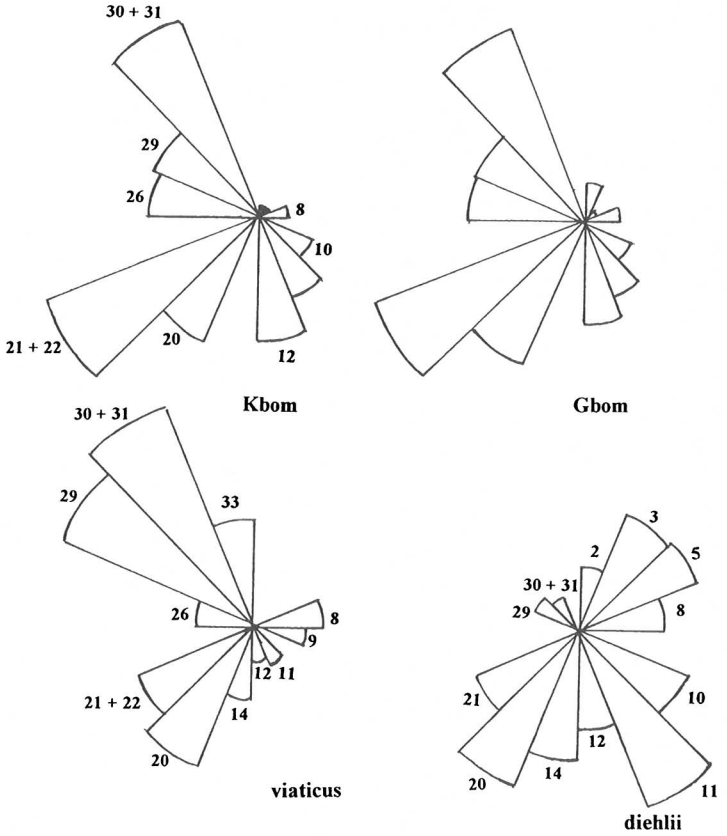

FIG. 3. Windroses for the mean amounts of the principal hydrocarbons of the postpharyngeal glands of two colonies of *Cataglyphis bombycinus* collected at Kebili (**kbom**) and Gafsa (**gbom**) in Tunisia, one colony of *Cataglyphis viaticus* (**bb**) from northern Tunisia and one of *Cataglyphis diehlii* (**diehl**) from southern Tunisia.

detected in any one sample. Some peaks consisted of more than one component (e.g., 9-methylheptacosane, 11-methylheptacosane, and 13-methylheptacosane eluted together as a single peak). Where there is a methyl branch near the middle of an alkyl chain the chromatographic column used is unable to separate the isomers. In the case of 7- and 9-methylnonacosane and 11,17-dimethylnonacosane, the peaks were sufficiently separated to integrate separately in some samples, but in others they were not, so these compounds have all been put together for representation on the windroses. In a few cases of branched alkanes that gave very weak mass spectra, it was not possible to determine the position of the methyl branch, so they are referred to as x-methylalkane. The closest resemblance in

postpharyngeal gland contents encountered was between **bdb** (*C. bicolor*) and **diehl**, but even here the differences were quite clear. *C. diehlii* lacked pentacosene (peak 1) and heptacosene (peak 9) and had much more of peak 20 and 21. The latter was absent from *C. bicolor*. It will be shown later that these two are also clearly separated by their Dufour gland contents (Gokçen et al., unpublished).

When all compounds had been identified, the 16 most abundant peaks from all the species were selected and the amounts of each of those peaks for a colony were plotted as windroses. The 16 compounds selected are shown in bold in Table 1 and are plotted on the windroses starting at 12 o'clock and moving clockwise around the diagram. The mean percentage composition of the secretion for each species is given in Table 1, while the mean percentage of the 16 selected compounds for each colony is plotted in Figures 1–3. The five colonies labelled **bdb** all fitted together with the same pattern (Figure 1) with a mixture of 9-, 11-, and 13-methylheptacosane (peak 11) as the largest component. The five samples of **bdw** (Figure 2) also had the same appearance when plotted as a windrose, with the mixture of 11- and 13-methylnonacosane (peak 20) as the largest component. The two samples called **kbom** and **gbom** were very similar to each other with components 21 + 22 and 30 + 31 clearly dominant. The remaining samples called **bb**, with peaks 29 and 30 + 31 very large, and **diehl**, with 11 and 20 largest, were all different (Figure 3).

DISCUSSION

In the northern part of Tunisia are found the *C. bicolor* group ants described here as **bb**, later defined as *C. viaticus*; then as one moves southward, there is a mixture of **bb** and **bdb**, now recognized as *C. bicolor*. Further south again, as the climate becomes drier, one finds only **bdb** or *C. bicolor*, and then a little further on a mixture of that and **bdw**, now defined as *C. savignyi*, which then continues southward to the Schotts (see Figure 7 in Wehner et al., 1994). Samples of **bdb** from each of the three areas in which it occurs (i.e., with **bb**, alone, and with **bdw**) have been analyzed. Chemically, **bdb** is still the same species in all these regions (see Figure 1). Included in the study were two colonies of the smaller species, *C. bombycinus*, not belonging to the *bicolor* group. These samples collected near Gafsa and Kebili and labeled **gbom** and **kbom** were included for comparison. They have already been eliminated from the *C. bicolor* group by their different mandibular gland secretion (Agosti et al., 1996; Gokçen et al., unpublished). Nevertheless they give clearly distinct patterns from the others, with peaks 21 + 22 and 30 + 31 most prominent (see Figure 3). Another species, *C. diehlii* belongs to the *bicolor* group but is significantly smaller than those labeled **bb**, **bdb** and **bdw**. *C. bicolor* and *C. savignyi*, which are more difficult

TABLE 1. AVERAGE PERCENTAGE COMPOSITION AND SAMPLE STANDARD DEVIATION (SD) FROM ALL SPECIMENS OF POSTPHARYNGEAL GLANDS FOR EACH SPECIES OF *Cataglyphis* ANT IN THIS STUDY

Peak number	Compound	*bicolor* **bdb** (N = 50)		*savignyi* **bdw** (N = 50)		*bombycinus* **bom** (N = 12)		*viaticus* **bb** (N = 10)		*diehlii* **diehl** (N = 10)	
		%	SD	%	SD	%	SD	%	SD	%	SD
1	Pentacosene	2.70	0.95								
2	**Pentacosane**	6.14	3.14			0.49	0.4			2.3	1.8
3	**11-Methylpentacosane**	6.21	1.87			T				8.4	3.7
	13-Methylpentacosane										
4	5-Methylpentacosane	2.30	0.67			T				1.2	0.7
5	**3-Methylpentacosane**	11.76	2.50			T		2.6	1.9	9.0	3.0
6	Hexacosane	3.88	0.80							2.4	1.4
7	x-Methylhexacosane	3.45	0.89							3.5	1.5
8	**4-Methylhexacosane**	1.86	0.64			0.51	0.4	1.4	1.0	4.3	1.3
9	**Heptacosene**	4.25	1.13								
10	**Heptacosane**	8.61	4.13	0.97	0.85	1.62	1.2			7.7	6.5
11	**9-Methylheptacosane**										
	11-Methylheptacosane	18.39	2.99	9.03	1.85	3.72	0.7	0.9	0.4	18.6	4.3
	13-Methylheptacosane										
12	**11,15-Dimethylheptacosane**	5.12	2.17	1.18	0.77	7.21	1.6	0.6	0.3	5.4	1.4
13	9,13-Dimethylheptacosane			1.23	0.40						
14	**3-Methylheptacosane**	5.57	1.57	4.57	1.35			2.9	1.2	8.7	3.0

15	Octacosane	2.69	1.16	1.28	0.63		T			1.0	0.8
16	13-Methyloctacosane	2.63	0.78	2.68	0.45	2.22	0.4		T	3.0	1.5
	16-Methyloctacosane										
17	4-Methyloctacosane	1.99	0.64	1.74	0.33	3.93	0.6	0.8	0.4	0.7	0.6
18	Nonacosene	0.93	0.45	2.59	0.52						
19	Nonacosane	0.94	0.64	2.29	1.92	2.09	1.5	1.2	1.0	0.7	0.6
20	**11-Methylnonacosane**										
	13-Methylnonacosane	6.20	1.84	26.39	3.33	11.38	2.1	12.5	2.6	15.0	5.6
	15-Methylnonacosane										
21	**7-Methylnonacosane**			18.25	3.84	5.50	0.9	8.7	1.9	6.8	4.7
	9-Methylnonacosane										
22	**11,17-Dimethylnonacosane**	2.62	0.78			21.76	1.7				
23	**3,9-Dimethylnonacosane**			8.87	3.05						
24	Triacontane			1.93	0.66						
25	Unknown hydrocarbon	1.13	0.54	2.09	0.44	2.83	0.6	3.0	0.3	0.9	0.7
26	**12-Methyltriacontane**			1.89	0.75	6.49	1.5	1.9	1.0		
27	**Hentriacontene**			2.47	1.30						
28	Unknown hydrocarbon			1.07	0.70						
29	**11-Methylhentriacontane**										
	13-Methylhentriacontane	1.27	0.60	5.87	1.30	7.04	0.8	24.4	1.1	1.3	1.1
30	**13,17-Dimethylhentriacontane**					23.60	2.8	30.2	6.8		
31	**5-Methylhentriacontane**	0.56	0.28	4.36	1.22					0.8	0.8
32	9-Methyltritriacontane							3.7	1.4		
	11-Methyltritriacontane										
33	**11,17-Dimethyltritriacontane**							6.5	1.7		

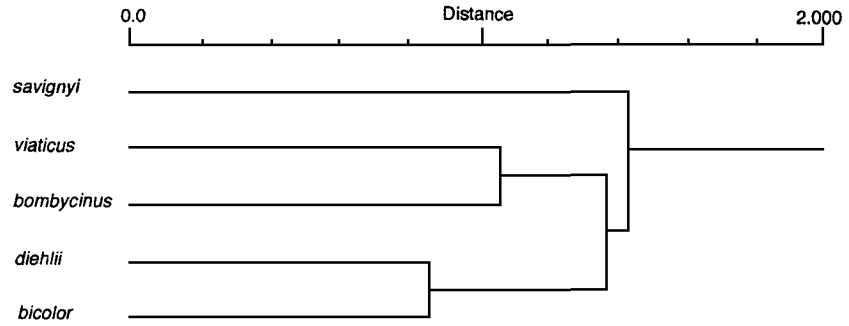

FIG. 4. Cluster analysis by the single-linkage method, using all the hydrocarbons of the postpharyngeal glands of the species of *Cataglyphis* studied here. Distance is the Euclidean distance.

to separate by their Dufour glands (Gokçen et al., unpublished), have distinctly different postpharyngeal gland contents (see Figures 1 and 2).

Cluster analysis was also performed on the total hydrocarbons of each species using the single linkage method (nearest neighbor), and separation of the species was clear (Figure 4). For example, *C. bicolor* and *C. diehlii* (which are morphologically distinct) are the most similar pair, with a separation distance of 0.863, *C. viaticus* and *C. bombycinus* form another pair of separation 1.096, but *C. bombycinus* is the one not belonging to the *bicolor* group. These pairs join at 1.370 and *C. savignyi* joins at 1.417. It is therefore very satisfying to find that the diagnosis of species based upon the difficult morphological criteria is supported completely by the chemical differences, and the results presented here strengthen the hypothesis that species can be defined by their chemical secretions.

Windrose diagrams, as used here, make a visual impact in pointing out these differences, in a much clearer way than a gas chromatogram does and more immediately than a table of statistical data. Twelve, 16, or 20 compounds can be conveniently plotted. We used the 16 most abundant compounds chosen from all the species, based on the mean values of these compounds for the species. We considered a statistical analysis of the data collected but found the differences between species was so clear that statistical treatment was not necessary. Similar diagrams have been used by Kaib et al. (1993) with equal success to illustrate differences in cuticular hydrocarbons of *Harpagoxenus sublaevis* and their slaves *Leptothorax* ants.

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COMPARATIVE STUDY OF MANDIBULAR GLAND SECRETIONS OF WORKERS OF PONERINE ANTS

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Abstract—The chemical contents of the mandibular glands of workers of eight species of ponerine ants have been analyzed and quantified. In three species (*Ectatomma* sp. from Brazil, *Odontomachus bauri*, and *Pachycondyla striata*) the contents were essentially a mixture of alkylpyrazines only. Three species (*Odontoponera transversa*, *Pachycondyla indica*, and *Pachycondyla obscuricornis*) contained a mixture of alkylpyrazines and oxygenated compounds and some simple terpenes; and the remaining two (*Diacamma vagans* and a *Diacamma* sp. from India) had only oxygenated compounds present. The two *Diacamma* species were notable in containing a dioxaspiroundecane (a spiroketal), not hitherto found in ants. All the 33 known mandibular gland analyses of workers of ponerine ant species have been brought together in order to seek some pattern in the type of glandular contents. Although the majority (24 species) produce alkylpyrazines, there is no observable pattern on a tribe or genus level.

Key Words—Hymenoptera, Formicidae, Ponerinae, mandibular gland, exocrine secretion, chemical analysis, alkylpyrazines.

INTRODUCTION

The large subfamily Ponerinae of the family Formicidae is regarded as a relatively primitive group of ants. This subfamily contains some of the most interesting and curious ants. Colony size varies considerably from as few as nine indi-

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viduals in some, e.g., *Pachycondyla* (*Bothroponera*) *sublaevis* (Peeters, 1990) to more than 52,000 in some *Leptogenys* sp. (Maschwitz et al., 1989). Some species have true queens while others have gamergates (mated, laying workers) (Peeters and Crewe, 1986). Workers of some species forage singly, some foragers recruit single individuals through tandem running and some practice mass recruitment, as in *Leptogenys nitida* (Duncan and Crewe, 1994). *Ectatomma ruidum* is said to practice cleptobiosis by locating the trails of other ant species to find workers carrying prey (Perfecto and Vandermeer, 1993). At least two species show a kind of primitive form of army ant behavior. *Pachycondyla marginata* in Brazil relocates its nest frequently and undertakes mass raids on termites (Leal and Oliveira, 1995), and a *Leptogenys* sp. nr. *mutabilis* in Malaysia forms large monogynous colonies, has frequent migrations, and makes mass raids in which all foraging activities are carried out collectively (Maschwitz et al., 1989).

Ponerine ants also appear to be chemically diverse, judged by the species that have been examined so far, in the products of their exocrine secretions. Blum and Hermann (1978) made the same point when only five species had been investigated. For example, some species have been found to produce a mixture of alkylpyrazines in their mandibular glands, but some species have only pyrazines while others contain mixtures with oxygenated compounds. Other ponerines produce simple alcohols and ketones in their mandibular glands, as do many ants from other subfamilies, but there is no discernible pattern. Other ponerines produce thioethers, some simple aromatic compounds, and some long-chain acids and derivatives.

Pyrazines are not confined to the ponerines, or to ants only, but in other ant subfamilies, pyrazines have generally been found in the poison glands (Attygalle and Morgan, 1984). However, there are exceptions. Alkylpyrazines have been found in the mandibular glands of *Myrmecia gulosa* (Myrmeciinae) (Brophy and Nelson, 1985), of *Wasmannia auropunctata* (Howard et al., 1982) and *Aphaenogaster rudis* (Wheeler et al., 1982) (both Myrmicinae), of *Calomyrmex splendidus* (Brown and More, 1979) and *Notoncus ectatommoides* (Brophy et al., 1982) (both Formicinae), and of *Iridomyrmex humilis* (now *Linepithema humile*) (Cavill and Houghton, 1974), *I. purpureus* (Cavill et al., 1985), *I. nitidus*, *I. rufoniger*, and *Dolichoderus clarkii* (Brophy, 1989) (all Dolichoderinae).

Brophy (1989) has reviewed the occurrence of pyrazines in insects, but the chemistry of the mandibular glands of ponerine workers has not yet been collated. We have collected all the available information on the exocrine chemistry of ponerine species, and because it still gives a fragmentary picture, we have taken advantage of opportunities to analyze further species to add to this information. We present here the accumulated data on mandibular gland chemistry and the analysis of eight further species. For the structure and naming of alkylpyrazines, see Figure 1.

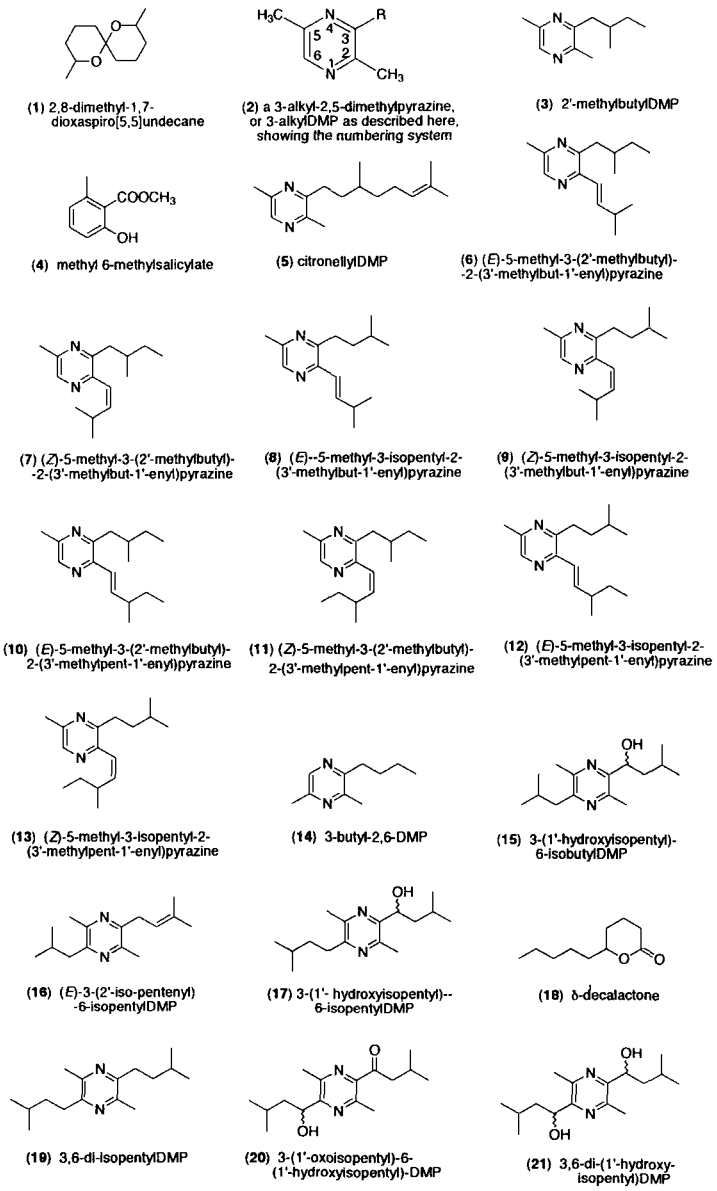

FIG. 1. Some of the substances found in the mandibular glands of ponerine ants, particularly the more complex pyrazines.

TABLE 1. LIST OF PONERINE SPECIES STUDIED WITH LOCATION OF THEIR COLLECTION

Tribe	Species, authority	Location, Country
Ectatommini	*Ectatomma* sp.	Maceió, AL, Brazil
Ponerini	*Diacamma vagans* (F. Smith, 1860)	Masinagudi, TN, India
	Diacamma sp.	Masinagudi, TN, India
	Odontoponera transversa F. Smith, 1857	Singapore
	Pachycondyla indica (Emery, 1899)	Bangalore, India
	Pachycondyla obscuricornis (Emergy, 1890)	Viçosa, MG, Brazil
	Pachycondyla striata F. Smith, 1851	Viçosa, MG, Brazil
Odontomachini	*Odontomachus bauri* Emery, 1892	Maceió, AL, Brazil

AL = Alagoas, TN = Tamil Nadu, and MG = Minas Gerais

METHODS AND MATERIALS

For all species studied, foraging workers were collected (Table 1). The majority of the mandibular contents were determined from the analysis of whole heads, as the mandibular glands are the only source of low-molecular-mass volatile compounds. Where necessary, mandibular glands were isolated by carefully removing the exoskeleton of the cephalic capsule, starting from the posterior side. In this way the mandible with the attached gland remained intact. Either the head or dissected mandibles were sealed in thin-walled glass capillaries according to the solid-sampling technique (Morgan and Wadhams, 1972; Morgan, 1990).

Gas chromatography–mass spectrometry was carried out on a Hewlett Packard 5890 Gas Chromatograph and 5970 Mass Selective Detector quadrupole mass spectrometer with 70 eV ionization. Chromatography was carried out on a fused silica capillary column (12 m × 0.32 mm with a 0.3-μm coating of polydimethylsiloxane; SGE, Milton Keynes, UK). Samples were heated in the injector to 150°C before crushing in a Keele solid-sample injector (Morgan, 1990). The oven was programmed from 30°C to 150°C at 8°C/min and then held isothermally. The carrier gas was helium at 1 ml/min.

Mass spectral identifications were confirmed wherever possible by comparison of retention times and mass spectra of synthetic standards. Where pure standards were not available, identification was based on comparison with spectra in our own collection and in computer libraries.

4-Methyl-4-hepten-3-one was prepared as described by Falk and Fry (1970), and 2,6-dimethyl-3-heptanol by the method of de Jong and Feringa (1991) and oxidized to the ketone with chromic acid.

TABLE 2. COMPOSITION OF MANDIBULAR GLANDS SECRETION OF *Diacamma vagans* AND *Diacamma* SPECIES WORKERS FROM INDIA (N = 3 EACH)

Component	Compound	Method of identification	Mean proportion (% ± SD)	
			D. vagans	*D.* sp.
1	2-Heptanone	RT, MS	3.0 ± 0.4	35.6 ± 4.9
2	3,4-Dimethyl-2-heptanone	RT, MS	0.7 ± 0.4	6.0 ± 1.2
3	5-Methyl-2-heptanone	RT, MS	0.5 ± 0.2	10.8 ± 0.7
4	2-Octanone	MS	0.5 ± 0.2	3.8 ± 0.6
5	3-Methyl-2-octanone	RT, MS	0.5 ± 0.1	1.3 ± 0.2
6	2-Nonanone	MS	0.6 ± 0.1	2.0 ± 0.8
7	3-Methyl-2-nonanone	MS	16.0 ± 4.4	6.6 ± 0.5
8	(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5,5]undecane	RT, MS	80.9 ± 4.0	33.9 ± 3.8
Mean total amount (ng)			89.0 ± 21.2	63.6 ± 10.8

RESULTS

Diacamma vagans *and a* Diacamma *sp.* Both species contained the same compounds, a mixture of ketones and a dioxaspiro-undecane (Table 2), in their mandibular glands, although in quite different proportions.

Ectatomma *Species.* The mandibular glands of the *Ectatomma* species, unidentified, possibly a new species, contained a mixture of seven pyrazines (Table 3). Two are of unknown structure but identical in mass spectra and of sim-

TABLE 3. COMPOSITION OF MANDIBULAR GLAND SECRETION OF *Ectatomma* SPECIES WORKERS FROM BRAZIL (N = 10)

Component	Compound	Method of identification	Mean proportion (% ± SD)
1	2,5-Dimethyl-3-ethylpyrazine	RT, MS	1.0 ± 1.7
2	2,5-Dimethyl-3-propylpyrazine	RT, MS	17.1 ± 10.5
3	2,5-Dimethyl-3-isobutylpyrazine	RT, MS	2.6 ± 2.3
4	2,5-Dimethyl-3-(2′-methylbutyl)pyrazine	RT, MS	56.5 ± 14.4
5	2,5-Dimethyl-3-pentylpyrazine	RT, MS	19.8 ± 9.8
6	Pyrazine of unknown structure with molecular formula $C_{16}H_{28}N_2O$	MS	1.6 ± 5.6
7	Unknown structure with molecular formula $C_{16}H_{28}N_2O$	MS	1.4 ± 4.3
Mean total amount (μg)			1.03 ± 0.90

TABLE 4. COMPOSITION OF MANDIBULAR GLAND SECRETION OF *Odontomachus bauri* WORKERS ($N = 10$)

Component	Compound	Method of identification	Mean proportion (% ± SD)
1	2,5-Dimethyl-3-ethylpyrazine	RT, MS	1.2 ± 2.9
2	2,5-Dimethyl-3-propylpyrazine	RT, MS	3.8 ± 3.1
3	2,5-Dimethyl-3-butylpyrazine	RT, MS	8.4 ± 15.9
4	2,5-Dimethyl-3-(2′-methylbutyl)pyrazine	MS	0.2 ± 0.4
5	2,5-Dimethyl-3-pentylpyrazine	RT, MS	73.9 ± 29.3
6	2,5-Dimethyl-3-hexylpyrazine	MS	7.9 ± 14.5
7	2,5-Dimethyl-3-(2′-methylhexyl)pyrazine	MS	0.2 ± 0.4
8	2,5-Dimethyl-3-heptylpyrazine	MS	4.7 ± 2.9
Mean total amount (μg)			1.62 ± 1.32

ilar relative retention to the two compounds with molecular formula $C_{16}H_{28}N_2O$ found by Tecle et al. (1987) in *Rhytidoponera metallica* from Australia.

Odontomachus bauri. Mandibular glands of workers of *Odontoponera bauri* contained a mixture of alkyl pyrazines, very similar to that of the *Ectatomma* species analyzed here, and as has been found in four other species of *Odontoponera* (see Table below). 2,5-Dimethyl-3-pentylpyrazine was by far the major component (Table 4).

Odontoponera transversa. Twenty individual glands of workers of *Odontoponera transversa* were analyzed. The secretion was found to consist of a mixture of oxygenated compounds and trisubstituted pyrazines (Table 5). The iso-

TABLE 5. COMPOSITION OF MANDIBULAR GLAND SECRETION OF *Odontoponera transversa* WORKERS ($N = 20$)

Component	Compound	Method of identification	Mean proportion (% ± SD)
1	4-Methyl-3-heptanone	RT, MS	29.4 ± 15.2
2	4-Methyl-3-heptanol	RT, MS	1.6 ± 1.6
3	2,6-Dimethyl-3-heptanone	RT, MS	1.0 ± 1.4
4	(*E*)-2-Isopropyl-5-methyl-2-hexenal	RT, MS	44.4 ± 19.1
5	(*Z*)-2-Isopropyl-5-methyl-2-hexenal	RT, MS	5.5 ± 3.7
6	2,5-Dimethyl-3-propylpyrazine	RT, MS	1.4 ± 1.0
7	2,5-Dimethyl-3-isobutylpyrazine	RT, MS	0.9 ± 0.9
8	2,5-Dimethyl-3-isopentylpyrazine	MS	0.4 ± 0.4
9	2,5-Dimethyl-3-pentylpyrazine	RT, MS	15.5 ± 4.1
Mean total amount (ng)			301.9 ± 262.1

TABLE 6. COMPOSITION OF MANDIBULAR GLAND SECRETION OF *Pachycondyla indica* WORKERS ($N = 2$)

Component	Compound	Method of identification	Mean proportion (%)
1	2,5-Dimethyl-3-ethylpyrazine	RT, MS	0.8[a]
2	2,5-Dimethyl-3-isobutylpyrazine	RT, MS	0.3[a]
3	2,5-Dimethyl-3-isopentyl-6(1′-hydroxyisopentyl)pyrazine	MS	1.3
4	Palmitoleic acid	RT, MS	1.2
5	Palmitic acid	RT, MS	10.6
6	Oleic acid	RT, MS	63.0
7	Stearic acid	RT, MS	22.5
8	Nonadecene	RT, MS	trace
9	Nonadecane	RT, MS	trace
Mean total amount (μg)			1.0

[a]Present in only one of the two specimens.

meric mixture of authentic isopropylhexenals used for confirmation of structure of components 4 and 5 were supplied by R. Lucas. These two components had earlier been found in trace quantities in the mandibular glands of *Dinoponera australis* (Oldham et al., 1994).

Pachycondyla *species.* Three species of *Pachycondyla* (*P. indica*, *P. obscuricornis*, and *P. striata*) have been added to the one species (*P. apicalis*) from Mexico already examined (Cruz López and Morgan, 1997). *P. indica* contained only traces of pyrazines and chiefly long-chain fatty acids (Table 6), while *P. obscuricornis* contained very volatile pyrazines (Table 7), and *P. striata* contained a complex mixture of 18 pyrazines (Table 8).

TABLE 7. COMPOSITION OF MANDIBULAR GLAND SECRETION OF *Pachycondyla obscuricornis* WORKERS ($N = 5$)

Component	Compound	Method of identification	Mean proportion (% ± SD)
1	Benzaldehyde	RT, MS	2.2 ± 1.6
2	2,5-Dimethylpyrazine	RT, MS	76.9 ± 13.7
3	Limonene	RT, MS	3.0 ± 3.6
4	Trimethylpyrazine	RT, MS	11.3 ± 13.0
5	2,5-Dimethyl-3-ethylpyrazine	RT, MS	5.0 ± 1.3
6	Indole	RT, MS	2.1 ± 3.4
Mean total amount (ng)			48.4 ± 34.8

TABLE 8. COMPOSITION OF MANDIBULAR GLAND SECRETION OF *Pachycondyla striata* WORKERS ($N = 5$)

Component	Compound	Method of identification	Mean proportion (% ± SD)
1	2,5-Dimethyl-3-ethylpyrazine	RT, MS	1.8 ± 0.9
2	2,5-Dimethyl-3-propylpyrazine	RT, MS	1.7 ± 0.4
3	2,5-Dimethyl-3-isobutylpyrazine	RT, MS	2.6 ± 1.9
4	2,5-Dimethyl-3-butylpyrazine	RT, MS	5.3 ± 1.5
5	2,6-Dimethyl-3-butylpyrazine	RT, MS	3.5 ± 0.7
6	2,5-Dimethyl-3-isopentylpyrazine	RT, MS	38.7 ± 4.3
7	2,5-Dimethyl-3-pentylpyrazine	RT, MS	9.0 ± 1.5
8	2,5-Dimethyl-3-isohexylpyrazine	MS	18.2 ± 4.9
9	2,5-Dimethyl-3-hexylpyrazine	MS	3.6 ± 0.8
10	2,5-Dimethyl-3-isoheptylpyrazine	MS	0.9 ± 0.4
11	2,5-Dimethyl-3-heptylpyrazine	MS	2.5 ± 0.4
12	2,5-Dimethyl-3-iso-octylpyrazine	MS	0.4 ± 0.2
13	2,5-Diemthyl-3-octylpyrazine	MS	2.4 ± 0.3
14	2,5-Dimethyl-3-isobutyl-6-(1′-hydroxypropyl)pyrazine	MS	0.4 ± 0.2
15	2,5-Dimethyl-3-isopentyl-6-(1′-hydroxypropyl)pyrazine	MS	0.3 ± 0.1
16	2,5-Dimethyl-3-isopentyl-6-(1′-hydroxybutyl)pyrazine	MS	0.6 ± 0.2
17	2,5-Dimethyl-3-isopentyl-6-(1′-hydroxyisobutyl)pyrazine	MS	0.5 ± 0.2
18	2,5-Dimethyl-3-isopentyl-6-(1′-hydroxyisopentyl)pyrazine	MS	2.4 ± 1.1
19	Octadecane	RT, MS	0.2 ± 0.1
20	Nonadecene	MS	1.8 ± 0.3
21	Nonadecane	RT, MS	1.0 ± 0.5
Mean total amount (ng)			1.8 ± 0.6

DISCUSSION

Diacamma is one of the ponerine genera that produce no true queens but the dominant reproductive individual is called a gamergate (Peeters and Crewe, 1986). It was interesting to find the principal compound in one species and one of the principal compounds in the other was a dioxaspiroundecane (structure **1**, Figure 1). This type of spiroketal has not been found before in ants, although it is common in the mandibular secretion of andrenid bees and polistine wasps. Both species contained the same mixture of simple ketones (Table 1).

Ectatomma is one of nine genera of the tribe Ectatommini, containing four

known species (*planidens*, *quadridens*, *ruidum*, and *tuberculatum*), all found in the Neotropics. The one collected here did not belong to these species. Besides the known alkylpyrazines, it contained two compounds identical in mass spectra and, as far as could be estimated, the same relative GC retention as two compounds found in an Australian species of *Rhytidoponera* (Tecle et al., 1987). Pratt (1990) reported that *E. ruidum* practices mass recruitment, and the mandibular gland contained an alarm pheromone.

Odontoponera transversa, the only species in this genus, is found throughout the Indo-Malayan region. Nothing has been reported of its behavior. Its mandibular glands contain three dominant alkylpyrazines, found in many other species.

Odontomachus, with over 100 species scattered throughout the tropics, are easily recognized by their specialized snapping mandibles. They are thought to navigate largely by visual clues (Oliveira and Hölldobler, 1989). Four species of *Odontomachus* have already been examined; they all, like *O. bauri*, contain a mixture of alkylpyrazines. The Odontomachini, with only six species examined, is the only tribe showing some uniformity, with only alkylpyrazines found in their mandibular glands.

Three species of *Pachycondyla*, all collected in India, were examined. They showed no great similarity in their mandibular gland chemistry. *P. indica* is the only ponerine species as yet known to have long-chain fatty acids in the gland. *P. obscuricornis* is unusual in having the very volatile dimethyl and trimethyl pyrazines, which do not seem to occur in any of the other pyrazine-dominated species. It has been recorded as one of the species that practices recruitment by tandem running (Hölldobler and Traniello, 1980). *P. striata* mandibular glands display the most complex mixture of pyrazines yet discovered and include a small quantity of alkanes.

There are 11 tribes of Ponerinae divided into 54 living genera. The contents of the mandibular glands of 33 species have now been examined (Table 9), but only three of these tribes and 17 genera are represented in the table. Yet a pattern might have been expected from that number. Of these 33 species, 20 contain only alkylpyrazines in their glands. A further four species contain some oxygenated compounds in addition to the pyrazines, while seven species contain volatile oxygenated compounds and two species (a *Megaponera* and a *Paltothyreus*, both from the Ponerini) contain sulfides and polysulfides. Of the six species from the Odontomachini, all produce alkylpyrazines. The structures of some of the more complex pyrazines are included in Figure 1.

The biosynthesis of pyrazines in insects has not been studied, but it is very likely that 2,5-dimethyl-3-alkylpyrazines are produced from the amino acid threonine, via a dimethyldihydropyrazine, which is condensed with an aldehyde to give a dimethylalkylpyrazine (Morgan, 1984). It is interesting to find that both citronellal and citronellyl DMP (5) are found together in *Rhytidoponera acicu-*

TABLE 9. CHEMICALS FROM MANDIBULAR GLANDS OF PONERINE ANTS

Tribe, genus, species	Chemicals[a]	Reference
Ectatommini		
Ectatomma sp.	Ethyl DMP, propyl DMP, isobutyl DMP, 2′-methylbutyl DMP (**3**), pentyl DMP, two pyrazines of unknown structure with molecular formula $C_{16}H_{28}N_2O$	This work
Gnamptogenus pleurodon	Methyl 6-methylsalicylate (**4**)	Duffield and Blum (1975)
Rhytidoponera aciculata	Citronellal, citronellic acid, citronellol, isopentyl DMP, citronellyl DMP (**5**)	Brophy et al. (1983)
Rhytidoponera chalybaea[b]	Isopentyl DMP	Brophy et al. (1981)
Rhytidoponera metallica	2-Methylbutyl DMP, isopentyl DMP, (*E*)- and (*Z*)-5-methyl-3-(2′-methylbutyl)-2-(3′-methylbut-1′-enyl)pyrazine, (**6**), (**7**) (*E*)- and (*Z*)-5-methyl-3-isopentyl-2-(3′-methylbut-1′-enyl)pyrazine, (**8**), (**9**) (*E*)- and (*Z*)-5-methyl-3-(2′-methylbutyl)-2-(3′-methylpent-1′-enyl)pyrazine, (**10**), (**11**) (*E*)- and (*Z*)-5-methyl-3-isopentyl-2-(3′-methylbut-1′-enyl)pyrazine, (**12**), (**13**) two pyrazines of unknown structure with molecular formula $C_{16}H_{28}N_2O$	Tecle et al. (1987)
Rhytidoponera victoriae	Isobutyl DMP, 2-methylbutyl DMP (**3**), isopentyl DMP, two pyrazines of unknown structure with molecular formula $C_{16}H_{28}N_2O$	Brophy (1989)
Ponerini		
Brachyponera sennaarensis	3-Butyl-2,6-DMP (**14**), 3-pentyl-2,6-DMP	Longhurst et al. (1978)
Diacamma vagans	3-Methyl-2-nonanone, (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5,5]undecane (**1**)	This work
Diacamma sp.	2-Heptanone, 3,4-dimethyl-2-heptanone, 5-methyl-2-heptanone, 2-octanone, 3-methyl-2-octanone, 2-nonanone, 3-methyl-2-nonanone, (*E, E*)-2,8-dimethyl-1,7-dioxaspiro[5,5]undecane (**1**)	This work
Dinoponera australis	Isobutyl DMP, 2-methylbutyl DMP (**3**), isopentyl DMP, 3-(1′-hydroxyisopentyl)-2,5-dimethyl-6-isobutylpyrazine (**15**), (*E*)-2,5-dimethyl-3-(2′-isopentenyl)-6-isopentylpyrazine (**16**), 3-(1′-hydroxyisopentyl)-2,5-dimethyl-6-isopentylpyrazine (**17**), and other minor products	Oldham et al. (1994)

Dinoponera grandis	Propyl DMP, isopentyl DMP	Hermann et al. (1984)
Euponera sp.	Isopentyl DMP	Brophy (1989)
Harpegnathos saltator	Isopentanol, 4-methyl-3-heptanone, 4-methyl-3-heptanol, 4-methyl-4-hepten-3-one, isobutyl 3-methylpentanoate	do Nascimento et al. (1993)
Hypoponera opacior	Isopentyl DMP	Duffield et al. (1976)
Megaponera foetens	Dimethyldisulfide, dimethyltrisulfide, benzyl methyl sulfide, ethyl methyl disulfide	Longhurst et al. (1979)
Odontoponera transversa	4-Methyl-3-heptanone, 4-methyl-3-heptanol, 2,6-dimethyl-3-heptanone, (*E*)-2-isopropyl-5-methyl-2-hexenal, (*Z*)-2-isopropyl-5-methyl-2-hexenal, propyl DMP, pentyl DMP	This work
Pachycondyla apicalis	Benzaldehyde, δ-decalactone (**18**)	Cruz López and Morgan (1997)
Pachycondyla[c] *castanea*	Isopentyl DMP, 6-isopentenyl-3-isopentyl DMP (three isomers), 3,6-di-isopentyl DMP 3-(1′-hydroxyisopentyl)-6-isopentyl DMP, 3-(1′-oxoisopentyl)-6-(1′-hydroxyisopentyl)-DMP, 3,6-di-(1′-hydroxyisopentyl)DMP (two isomers, *meso* and *erythro* forms)	Fales et al. (1984, 1988)
Pachycondyla[c] *castaneicolor*	Isopentyl DMP, (like *P. castanea*, but no details given)	Fales et al. (1984, 1988)
Pachycondyla indica	Palmitic acid, stearic acid, oleic acid	This work
Pachycondyla obscuricornis	Benzaldehyde, DMP, limonene, trimethylpyrazine, ethyl DMP, indole	This work
Pachycondyla soror[d]	(Unidentified), 2-undecanone, (a methyl ester), 2-undecanol, 2-undecenol (two isomers), 2-dodecanol, methyl 5-methylsalicylate, 2-tridecanol, methyl 6-methylsalicylate	Longhurst et al. (1980)

TABLE 9. CONTINUED

Tribe, genus, species	Chemicals[a]	Reference
Pachycondyla striata	Ethyl DMP, propyl DMP, butyl DMP, butyl-2,6-DMP, isobutyl DMP, pentyl DMP, isopentyl DMP, hexyl DMP, isohexyl DMP, heptyl DMP, octyl DMP, 3-(1′-hydroxyisopentyl)-2,5-dimethyl-6-isopentylpyrazine, nonadecene, nonadecane	This work
Pachycondyla villosa[e]	4-Methyl-3-heptanone, 4-methyl-3-heptanol	Duffield and Blum (1973)
Paltothyreus tarsatus	Dimethyldisulphide, dimethyltrisulphide	Casnati et al. (1967), Crewe and Fletcher (1974)
Ponera pennsylvanica	Isopentyl DMP	Duffield et al. (1976)
Streblognathus aethiopicus	Butyl DMP, pentyl DMP, hexyl DMP	Jones et al. (1998)
Odontomachini		
Anochetus sedilloti	3-s-Butyl-2,6-DMP	Longhurst et al. (1978)
Odontomachus bauri	Ethyl DMP, propyl DMP, butyl DMP, pentyl DMP, hexyl DMP, heptyl DMP	This work
Odontomachus brunneus	Ethyl DMP, propyl DMP, butyl DMP, pentyl DMP	Wheeler and Blum (1973)
Odontomachus clarus	Isopentyl DMP	Wheeler and Blum (1973)
Odontomachus hastatus	Isopentyl DMP	Wheeler and Blum (1973)
Odontomachus troglodytes	3-Butyl-2,6-DMP, 3-pentyl-2,6-DMP	Longhurst et al. (1978)

[a]DMP: dimethyl pyrazine. Unless otherwise stated, the substitution is 3-(substituent)-2,5-dimethylpyrazine.
[b]Originally called *R. metallica* but corrected to *R. chalybaea* by Brophy et al. (1983).
[c]*Pachycondyla* (= *Mesoponera*).
[d]*Pachycondyla* (= *Bothroponera*).
[e]*Pachycondyla* (= *Neoponera*).

lata (Brophy et al., 1983). Tetrasubstituted alkylpyrazines, found so far only in *Dinoponera australis* and three species of *Pachycondyla* —*P. castanea*, *P. castaneicolor*, and *P. striata* (Table 9)—are probably derived from the trisubstituted pyrazines by further alkylation (Oldham and Morgan, 1993). 2,6-Dimethyl-3-alkylpyrazines have been identified too many times for them to be misidentifications; they appear in four species of two tribes in Table 9. There seems to be no clear relation between the biosynthetic origin of the oxygenated compounds and the alkyl groups of the pyrazines except in the special cases of *Rhytidoponera aciculata* (above) and some minor products of *Dinoponera australis* (Oldham and Morgan, 1993). The oxygenated compounds found here are derived from acetate and propionate via acetogenin methods. 4-Methyl-3-heptanone and 4-methyl-3-heptanol, compounds common in other ant mandibular glands, appear in three species in the Ponerini but a variety of 2-alkanones are found in other ponerines.

For the great majority of these volatile secretions, we have no information about their communicative function. In early reports and more recently that by Pratt (1990), the mandibular glands of ponerines are said to be the source of alarm pheromones. In the one example we examined carefully, *Dinoponera australis*, we found no evidence of alarm generated by the alkylpyrazines of the mandibular glands (Oldham et al., 1994). From observations of behavior, we suspect this species uses the secretion to mark the area near the nest entrance, but we have no firm evidence.

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A NOVEL APPROACH FOR ISOLATION OF VOLATILE CHEMICALS RELEASED BY INDIVIDUAL LEAVES OF A PLANT IN SITU

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Abstract— A glass chamber designed specifically for collecting volatile chemicals from individual leaves of a plant in situ is described. The effectiveness of the chamber was demonstrated by collecting volatile chemicals from single leaves of two plant species, potato (*Solanum tuberosum*) and broad bean (*Vicia faba*), before and after mechanical damage. The glass chamber, in conjunction with thermal desorption, enables reduction of the entrainment time and thereby allows the monitoring of compounds released by leaf damage in successive 5-min periods. An intact broad bean leaf, in the middle of the day, produces small amounts of the green leaf volatiles (*E*)-2-hexenal and (*Z*)-3-hexen-1-ol. However, during the first 5 min after mechanical damage, large amounts of (*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol are produced. The decline in production of (*Z*)-3-hexenal and (*E*)-2-hexenal is fast, and after 10 min, these compounds reach very low levels. (*Z*)-3-Hexen-1-ol shows an increase for the first 10 min and then a gradual decline. An intact potato leaf, in the middle of the day, produces very small amounts of the sesquiterpene hydrocarbons β-caryophyllene and germacrene-D. After being damaged, the profile of released volatiles is different from that of broad bean. In potato, damage is associated with release of large amounts of green leaf volatiles and sesquiterpene hydrocarbons. Compounds such as (*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol are released in high amounts during the first 5 min after damage, but after 10 min, these drop to very low levels. High release associated with damage is also observed for β-caryophyllene, (*E*)-β-farnesene, germacrene-D, and β-bisabolene. The highest level is reached 5 min after damage and 15 min later, these compounds drop to low levels. The significance of compounds released after plant damage is discussed.

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Key Words—Volatile collection chamber, mechanical leaf damage, *Solanum tuberosum*, *Vicia faba*, sesquiterpenes, green leaf volatiles.

INTRODUCTION

Volatile plant semiochemicals mediate a wealth of interactions between organisms from different trophic levels, including phytophagous insects and their parasitoids and predators (Vet and Dicke, 1992). In studies of insect–plant interactions, it is essential to identify and quantify the chemicals mediating those interactions, to elucidate the factors affecting their release, and to obtain information on the time course of their emission. The techniques used to collect volatile plant chemicals should provide all the above information without disturbing the plant. Time is very important in all plant studies, because most chemicals show a time-dependent release profile (Loughrin et al., 1990). At present, the techniques used to monitor plant compounds require relatively long collection periods and are thus unable to provide precise information on the time course of release of compounds emitted over short periods of time. Furthermore, the handling of plant material before and during the collection of volatile chemicals is crucial. Techniques requiring maceration or chopping of the plant material, followed by extraction and distillation, allow enzymatic and chemical reactions to occur that cause changes in the volatile composition of the sample (Blight, 1990). Although such procedures are useful for the isolation of large amounts of specific chemicals, they do not provide information on what is emitted by a living plant. For example, Tollsten and Bergström (1998) showed that intact plants of a number of *Brassica* species released a different blend of volatile chemicals to macerated plant material. Practices that involve cut stems, detached leaves, or uprooted plants placed in water (Dicke et al., 1990; Whitman and Eller, 1990; Croft et al., 1993; Loughrin et al., 1995; Takabayashi et al., 1995; Geervliet et al., 1997) may also alter the chemistry of the plant. Mookherjee et al. (1989) demonstrated that the profile of volatiles emitted by cut flowers of rose, narcissus, and osmanthus and by cut stems of spearmint was different from that of intact flowers and plants. Additionally, mechanical injury of plant tissue induces changes in the chemistry of the intact parts of the plant (Paré and Tumlinson, 1997). To overcome these factors, researchers have used potted plants enclosed in large entrainment chambers, thus avoiding injury to the plant (Cole, 1980; Panasiuk, 1984; Blaakmeer et al., 1994; Bolter et al., 1997). In such systems, the plant suffers no physical injury, but contaminants released from the pot, soil, or microorganisms inhabiting the soil may mask part of, or contribute to, the volatile profile recorded. The problem of isolating the pot from the aerial parts of the plant has been overcome by using specially designed entrainment chambers made of solid (e.g., glass) and soft materials (plastic bags) (Loughrin et al.,

1990; Heath and Manukian, 1992, 1994; Agelopoulos and Keller, 1994; Jakobsen and Olsen, 1994; Tollsten and Müller, 1996). Although in situ collection of foliage volatiles has been achieved, no information has been obtained on the volatile profile released by single leaves of a plant in situ. Information related to volatile chemicals emitted by different leaves of a plant before and after injury has been reported only from detached leaves (Takabayashi et al., 1994).

Here, a new glass chamber is described that has been designed specifically for the collection of volatile chemicals released from single leaves of a plant in situ. The use of the chamber is demonstrated by collecting volatile chemicals from single leaves of two plant species, *Vicia faba* (broad bean) and *Solanum tuberosum* (potato), before and after mechanical injury. The leaf chamber, in conjunction with thermal desorption, in which all the trapped volatiles are introduced onto the chromatographic column, enabled monitoring of compounds released after leaf damage over periods of 5 min.

METHODS AND MATERIALS

Plants. Broad bean, *Vicia faba* L. cv. Sutton (Fabaceae), and potato, *Solanum tuberosum* L. cv. Desirée (Solanaceae), were grown in a greenhouse at 14–18°C.

Leaf Chamber and Collection of Volatile Chemicals. The leaf chamber (Figure 1) was constructed from Pyrex glass and consisted of a male and a female part. The dimensions of the chamber when closed were as follows: 5.8 cm high, internal diameter 4.2 cm ID, approximately 80 ml volume. The female part (4 cm high; 5 cm ID) had an air inlet and a ground glass connection (2 cm long). The male part (4.4 cm high; 4.2 cm ID) had a ground glass connection (2 cm long), an outlet for the air, and a slot (2.5 cm long; 3 mm wide) that ran through and extended 5 mm beyond the ground glass connection. The petiole of the leaf passed through the slot of the male part and the two parts were then joined together in the ground glass connection, leaving an opening 5 mm long and 3 mm wide through which the petiole passed. The slot was partially sealed with Teflon tape. Volatiles were adsorbed on Tenax TA (50 mg) (Supelco) contained in a glass tube (8 cm long; 3 mm ID) (ATAS, Cambridge, UK). Before use, the Tenax was washed with diethyl ether and then conditioned at 160°C for 10 hr in a stream of nitrogen. The entrainments were performed by using a positive/negative pressure system in which clean air, purified by passage through molecular (10 Å) and charcoal (10–18 mesh) sieves, was pumped into the chamber at a flow of 35 ml/min (positive pressure). Air was simultaneously drawn from the chamber through the Tenax trap by a mini pump at 30 ml/min (negative pressure) (NMP 08 S, 6V, ATAS). All entrainments were performed in a temperature controlled room (18–20°C). Because of the small size of the col-

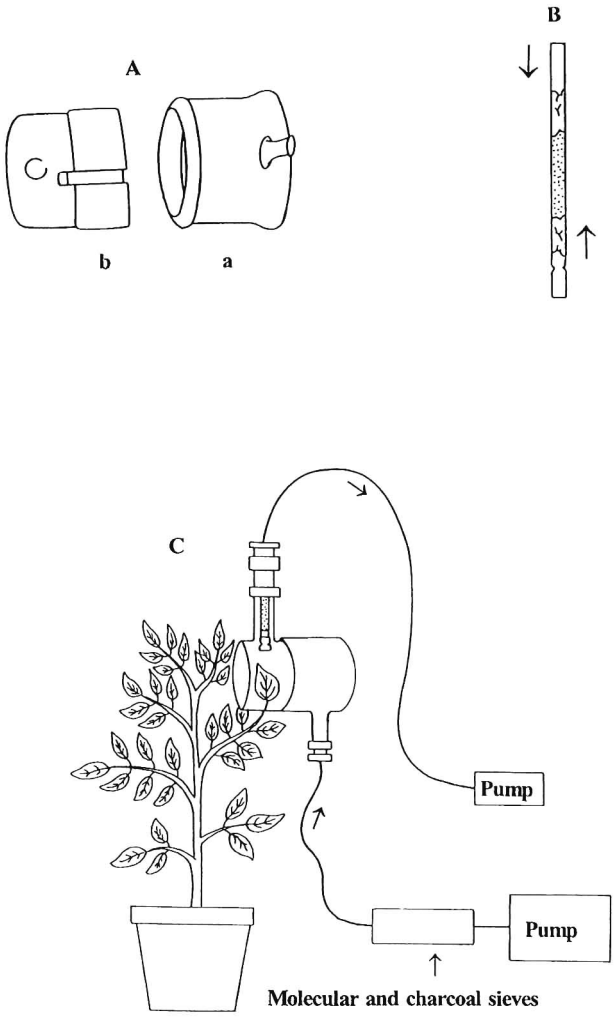

FIG. 1. A: The leaf chamber, female (a) and male part (b). B: Tenax packed thermal desorption liner with top arrow showing the direction of the carrier gas and bottom arrow the way that the internal standard was injected. C: Schematic presentation of an entrainment of volatile chemicals released by a single potato leaflet in the leaf chamber.

lection chamber, the glass tube holding the polymer could be positioned close to the leaf surface (approximately 5 mm from the leaf). While entraining chemicals released by damage, the Tenax trap was located immediately above the damaged part of the leaf.

Experimental Procedure. Volatile chemicals were collected in situ (Figure 1), in the middle of the day, from the fourth leaf (counting from the bottom) of a broad bean plant at the stage of 5 fully expanded leaves, and from the top leaflet of the ninth leaf (counting from the bottom) of a potato plant at the stage of 10 fully expanded leaves. Volatiles were desorbed from the Tenax by thermal desorption or liquid elution. Samples for thermal desorption were collected from intact and damaged leaves. Entrainments were performed on an intact leaf for 30 min. At the end of the entrainment, the same leaf was mechanically damaged by puncturing four round holes (7 mm diameter) through the surface. Immediately after damage, the volatiles were collected every 5 min for 15 min (0–5 min, 5–10 min, and 10–15 min). Two samples, one from potatoes and one from beans, relating to volatile chemicals released during the first 5 min after damage, were obtained for liquid elution by using the entrainment system described above. For potatoes, a collective sample was obtained by entraining the top leaflet of the ninth leaf of six different plants in succession. For broad beans, a collective sample was obtained by entraining the fourth positional leaf of six plants in succession. On each leaf, entrainments were performed for 5 min immediately after damage. After the end of the collective entrainment, the Tenax TA tube was eluted with 500 μl of redistilled diethyl ether, which was then concentrated to 50 μl under a slow flow of nitrogen.

Gas Chromatography (GC). The samples were analyzed on an AI 94 model gas chromatograph fitted with an Optic 400 programmed temperature vaporization injector (PTV) (ATAS), a polar column HP-Wax (30 m $\times$ 0.32 mm ID, 0.5-μm film thickness) (Hewlett Packard), and a flame ionization detector (FID). Desorption inside the PTV unit was performed with a rapid temperature ramp starting at 30°C (temperature of the PTV injector during introduction of the sample) and reaching 220°C in 40 sec. The oven temperature was held at 30°C for 5 min and then programmed at 10°C/min to 220°C. The carrier gas was nitrogen. For thermal desorption samples, quantification was achieved by injecting, at the bottom of the liner (before the introduction of the liner into the PTV unit), 1 μl (100 ng/μl) of the internal standard decane, which was not present in the volatile profile of beans or potatoes. The liquid desorption samples were injected into an empty liner of the PTV unit and quantification was achieved by adding 1 μl (100 ng) of the internal standard into the sample.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). Compounds were tentatively identified by coupled gas chromatography–mass spectrometry (GC-MS) (VG Autospec, Fisons). Ionization was by electron impact at 70 eV, 250°C. The HP-1 GC column (50 m $\times$ 0.32 mm ID, 0.52-μm film thickness; Hewlett Packard) was maintained at 30°C and then programmed at 5°C/min to 250°C. For the liquid sample, the identity of the compounds was confirmed by coinjections with authentic samples on both polar SPB-35 (30 m $\times$ 0.32 mm ID, 0.25-μm film thickness; Supelco) and nonpolar HP-1 (50 m $\times$ 0.32 mm

ID, 0.52-μm film thickness; Hewlett Packard) columns. The compounds germacrene-D and β-bisabolene were tentatively identified from GC-MS data alone because no authentic samples were available. Synthetic compounds were obtained from commercial sources (Aldrich Chemical Co. Ltd ; Fluka Chemie AG; Koch-Light Laboratories Ltd.; Lancaster Synthesis Ltd.) and were ≥97% pure. (*Z*)-3-Hexenal (90% pure) was synthesized as described by Kajiwara et al. (1975) and verified as the *Z* isomer by a coupling constant of 10.7 Hz between the alkene protons in the proton NMR spectrum (Jeol GX400 FT NMR spectrometer).

RESULTS AND DISCUSSION

Leaf Chamber. The design of the leaf chamber allowed the isolation of a single leaf of a plant in situ and the analysis of compounds with rapid release profiles related to plant physical injury. The short entrainment periods (5 min) were achieved by the small size of the chamber, allowing the sorbent trap to be placed very close to the leaf surface, and the use of thermal desorption. The positive–negative pressure system was used to avoid build-up of pressure inside the entrainment chamber. The differential flow rates (35 ml/min purge; 30 ml/min trap) used in the positive-negative entrainments ensured that there was no back diffusion of volatiles from laboratory air into the system, and blank entrainments showed no evidence of such contaminants. The chamber can be utilized for studying the headspace composition of a variety of samples by modifying its size and the position of the sorbent trap. Although originally designed for in situ headspace analysis of single leaves, a modified system is currently being used for headspace analysis of whole plants and flowers in situ.

Analysis by GC. The small size of the leaf chamber has allowed a closer approach to the leaf surface, but the short entrainment periods would not have been achieved without the use of thermal desorption. The volatiles trapped on the polymer can be removed by using a solvent (solvent elution/solvent desorption) or by rapid heat treatment (thermal desorption). Thermal desorption has two main advantages over liquid elution. First, the sample can be introduced into the GC, thus increasing the sensitivity and allowing reduced entrainment time and, secondly, there is no solvent peak to mask components with short retention times (Agelopoulos and Pickett, 1998). Although it provides greater sensitivity, thermal desorption is not without drawbacks, particularly the thermal instability of some compounds and use of the entire sample in the chromatographic analysis. At present, the solvent peak is the major disadvantage of solvent elution, since large-volume injections of liquid samples are already possible (Munari et al., 1995; Grob and Biedermann, 1996). This study employed both thermal and solvent desorption. Thermal desorption was used to achieve short entrain-

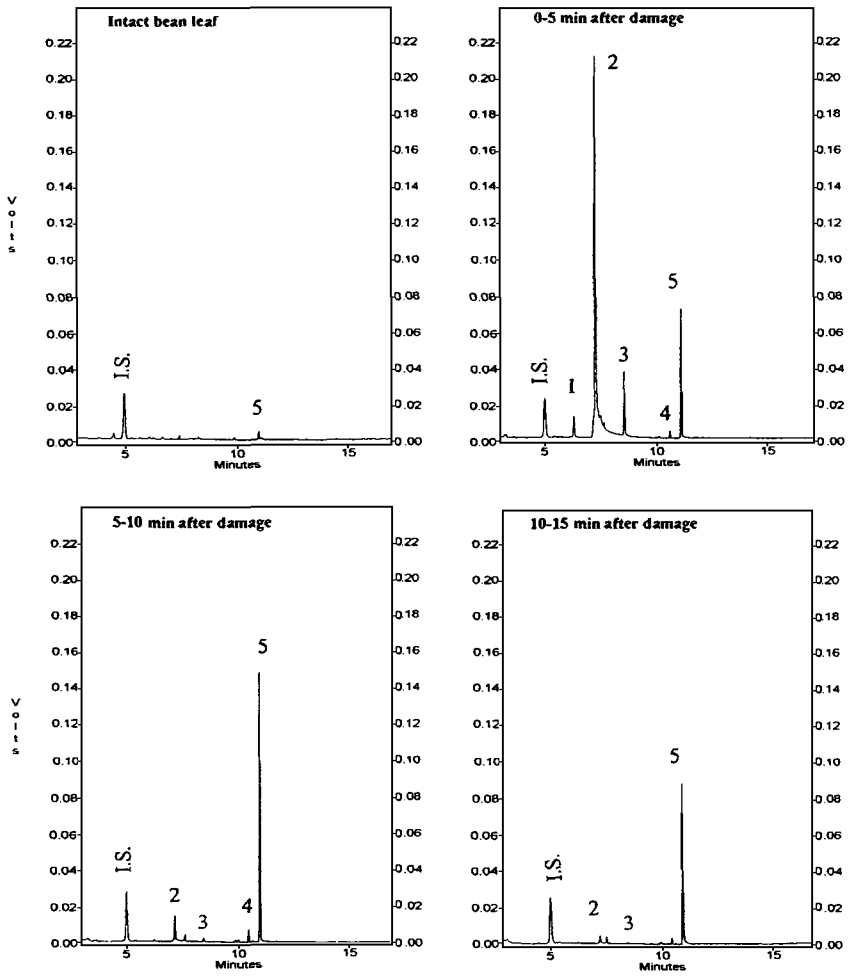

FIG. 2. GC profiles of an intact and mechanically damaged broad bean leaf on the HP-Wax polar column. Entrainments were performed for 30 min on an intact leaf and every 5 min for a period of 15 min after mechanical damage. The compounds were identified by coinjections with authentic samples: (1) hexanal, (2) (*Z*)-3-hexenal, (3) (*E*)-2-hexenal, (4) hexan-1-ol, (5) (Z)-3-hexen-1-ol. I.S. (internal standard) is decane (100 ng).

ment periods and thus provided information on the release profile of compounds (Figures 2 and 3), while solvent desorption provided samples for identification (coinjections with authentic samples) and for the validation of the thermal desorption samples (Figure 4). In chemical ecology studies, a liquid sample has

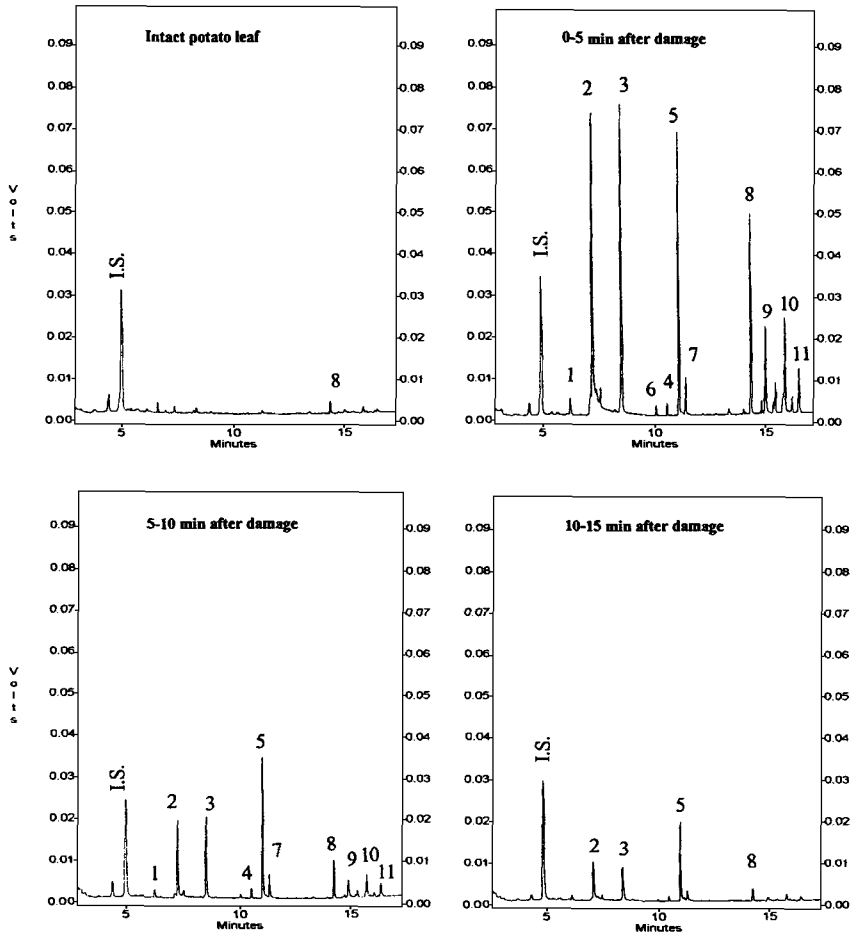

FIG. 3. GC profiles of an intact and mechanically damaged potato leaf on the HP-Wax polar column (30 m long, 0.32 mm ID, 0.50-μm film thickness). Entrainments were performed for 30 min on an intact leaf and every 5 min for a period of 15 min after mechanical damage. (1) Hexanal, (2) (*Z*)-3-hexenal, (3) (*E*)-2-hexenal, (4) hexan-1-ol, (5) (*Z*)-3-hexen-1-ol, (6) (*Z*)-2-penten-1-ol, (7) (*E*)-2-hexen-1-ol, (8) β-caryophyllene, (9) (*E*)-β-farnesene, (10) germacrene-D, and (11) β-bisabolene. I.S. (internal standard) is decane (100 ng).

advantages because it can be used a number of times, allowing replication, such as for bioassays, electrophysiology, or analysis on another GC column.

Plant Response to Mechanical Damage. Qualitative and quantitative differences were observed in the volatile profiles emitted by leaves of potatoes and

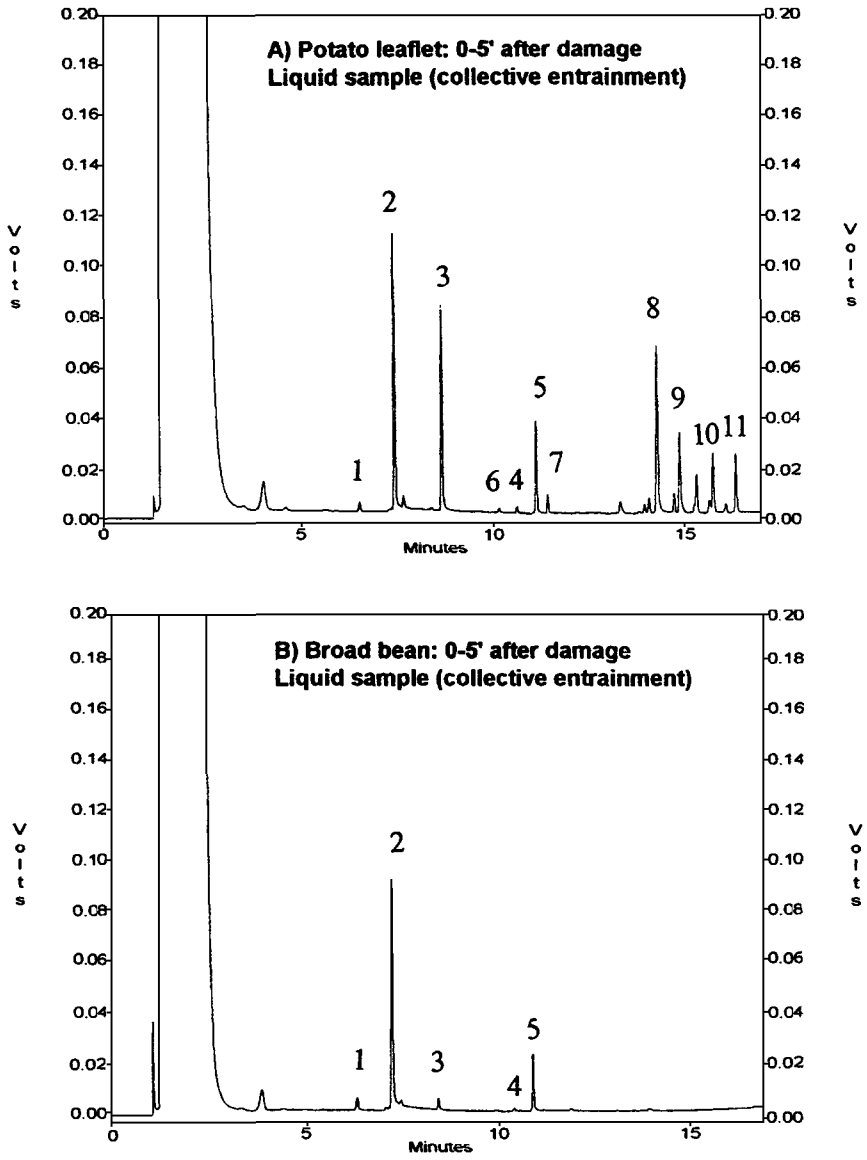

FIG. 4. Chromatographic profile of collective entrainments of potato leaves (A) and broad beans (B) after solvent desorption. (1) Hexanal, (2) (*Z*)-3-hexenal, (3) (*E*)-2-hexenal, (4) hexan-1-ol, (5) (*Z*)-3-hexen-1-ol, (6) (*Z*)-2-penten-1-ol, (7) (*E*)-2-hexen-1-ol, (8) β-caryophyllene, (9) (*E*)-β-farnesene, (10) germacrene-D, and (11) β-bisabolene.

broad beans before and after mechanical damage. At midday, the intact fifth leaf of a bean produced only small amounts of (*Z*)-3-hexen-1-ol and (*E*)-2-hexenal (Figure 5). The profile of emitted volatiles changed dramatically after mechanical damage to the leaf, with high levels of (*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-3-Hexen-1-ol, and small quantities of hexanal and 1-hexanol being produced in the first 5 min after damage. In the next 10 min, there was a rapid decline in the production of both (*Z*)-3-hexenal and (*E*)-2-hexenal. (*Z*)-3-Hexen-1-ol showed an increase for the first 10 min, and after 15 min was still released in high amounts when compared to the two aldehydes (Figures 2 and 5). The airflows used in the current study allowed the air in the entrainment vessel to be changed approximately twice during each 5-min sampling period. Higher turnover rates, and shorter entrainment periods, may show that the release of compounds associated with mechanical damage is faster than reported here.

An intact potato leaflet at midday produced very small amounts of sesquiterpene hydrocarbons (Figure 5). In contrast to broad beans, mechanical damage to a potato leaf was manifested by release of large amounts of both green leaf volatiles and sesquiterpene hydrocarbons (Figures 3 and 5). With the exception of (*Z*)-2-penten-1-ol and (*E*)-2-hexen-1-ol, most of the green leaf volatiles released by damaged potatoes were also present in broad beans. As with broad beans, the major green leaf volatiles (*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol, were released in high amounts during the first 5 min after damage, followed by a rapid decline in production of (*Z*)-3-hexenal and (*E*)-2-hexenal. The production of (*Z*)-3-hexen-1-ol in potatoes was different from that of broad beans. In potatoes, it was released in high amounts for the first 5 min after damage and then declined gradually, although not as fast as (*Z*)-3-hexenal and (*E*)-2-hexenal. The production of the same green leaf volatiles by both broad beans and potatoes is due to the universal reactions taking place upon physical injury of plant tissue (Galliard, 1978). During physical wounding, the integrity of cell membranes is disrupted, resulting in loss of cellular compartmentalization, mixing of substrates, and release/activation of enzymes (Bostock and Stermer, 1989; Davies, 1987). Membrane lipids are attacked by acyl hydrolases activated because of the injury, resulting in free fatty acids, which produce green leaf volatiles by the action of lipoxygenases. Although mechanical damage to leaves results in the production of similar green leaf volatiles, there can be, as shown by this study, very significant quantitative and qualitative differences between species (Figure 5).

In the intact state at midday, the sampled potato leaflet released small amounts of sesquiterpenes. Upon damage, the amount and number of sesquiterpene hydrocarbons that were released increased significantly (Figure 3), including compounds such as β-caryophyllene, (*E*)-β-farnesene, germacrene-D, and β-bisabolene. The latter two compounds were identified from MS data alone because no authentic samples were available. In addition, a number of other

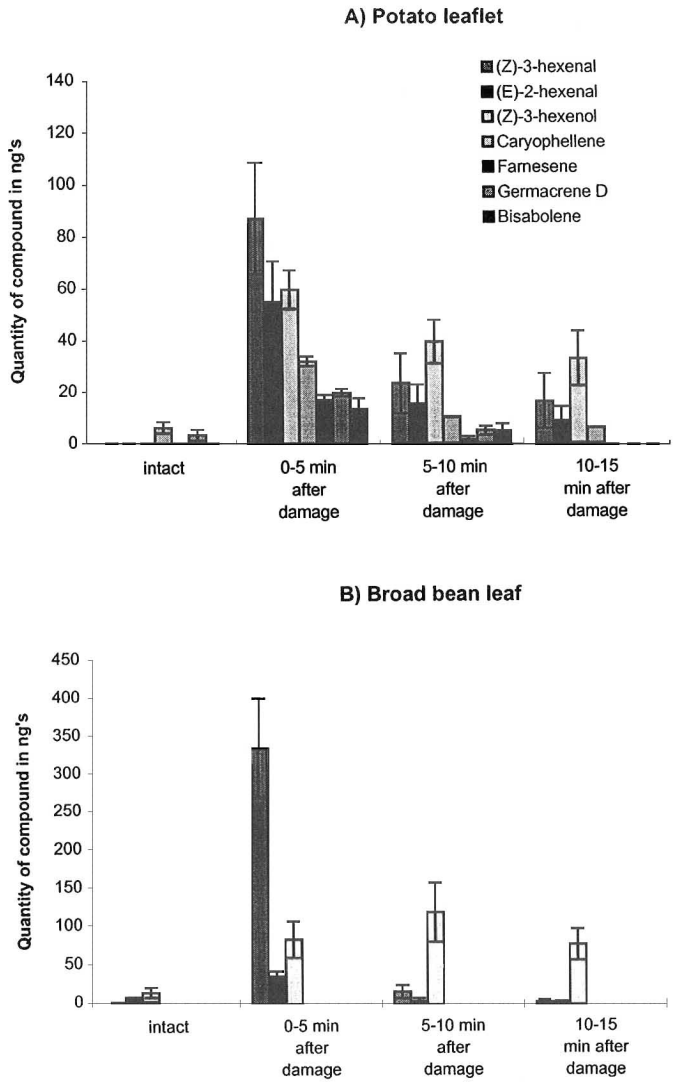

FIG. 5. Release profile of compounds produced by intact and mechanically damaged potato leaflet (A) and a broad bean leaf (B).

sesquiterpenes, not yet fully characterized, were released during damage. Release mirrored that of the green leaf volatiles, with the highest level 5 min after damage and minimal levels reached 15 min after damage (Figure 5). Although the amount for all sesquiterpene hydrocarbons was high and the proportions released

were typical of mechanical damage, the presence of these compounds does not appear to be related to mechanical damage, as they are also present in intact potato leaves.

Many of the volatile chemicals released immediately after damage have been shown to be involved in volatile defense against phytopathogenic bacteria, fungi, and phytophagous insects. For example, volatiles from crushed tomato leaves inhibited the growth of the fungi *Alternaria alternata* and *Botrytis cinerea* (Hamilton-Kemp et al., 1992), and volatiles from wounded cotton leaves reduced the growth of the fungus *Aspergillus flavus* (Zeringue and McCormick, 1989). Volatiles from crushed tomato and tobacco leaves reduced progeny numbers of the aphid species *Myzus nicotianae* (Hildebrand et al., 1993).

Pure samples of green leaf volatiles have been tested to ascertain their effects on natural enemies of plants. At very low concentrations, (*E*)-2-hexenal has high antibacterial (Croft et al., 1993) and antifungal properties (Lyr and Banasiak, 1983; Zeringue and McCormick, 1989; Hamilton-Kemp et al., 1992), while at high concentrations, (*Z*)-3-hexen-1-ol has antibacterial (Croft et al., 1993) and antifungal properties (Zeringue and McCormick, 1989). Information on the effects of (*Z*)-3-hexenal is lacking because of its chemical instability and lack of commercial availability. Because of their role against phytopathogens, the green leaf volatiles have been referred to as "volatile phytoalexins" (Lyr and Banasiak, 1983; Hamilton-Kemp et al., 1992). A number of essential oils have also been shown to have bactericidal and fungicidal properties (Sivropoulou et al., 1996, 1997; Langenheim, 1994). The insecticidal properties of some terpenoids have been shown against insect pests of stored products (fumigant toxic effect) (Regnault-Roger and Hamraoui, 1995; Regnault-Roger et al., 1993; Shaaya et al., 1991) and on larvae of *Drosophila* spp. (Konstantopoulou et al., 1992; Franzios et al., 1997). Sesquiterpenes and lower terpenes also show sublethal and behavioral effects on aphids (Gutiérrez et al., 1997; Hildebrand et al., 1993), and sesquiterpenes such as α-humulene and β-caryophyllene, present in tomato trichomes, are toxic to the larvae of *Spodoptera exigua* (Eigenbrode et al., 1994).

The rapid production of relatively large amounts of chemicals released on leaf damage with bactericidal, fungicidal, and insecticidal properties and their fast decline suggests a direct volatile defense against enemies already present on the plant surface and ready to invade the plant tissues. Compounds released immediately after damage are also involved in other types of interactions such as attraction of phytophagous insects and their natural enemies (Vet and Dicke, 1992), and may also mediate plant inter- and intraspecific interactions. Information on the release profile of such compounds will enable us to understand more fully their ecological roles.

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EFFECTS OF HERBIVORE DAMAGE AND NUTRIENT LEVEL ON INDUCTION OF IRIDOID GLYCOSIDES IN *Plantago lanceolata*

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Abstract—Damage by larvae of the buckeye butterfly (*Junonia coenia*) resulted in removal of 15–25% of *Plantago lanceolata* leaf area. Plants grown under high nutrients were larger than those grown under low nutrients. Twenty-eight days after herbivory, plants grown under high nutrients were still larger than those grown under low nutrients, and plants exposed to herbivores were significantly smaller than those not exposed to herbivores, regardless of the nutrient treatment. Damage by larvae also increased the iridoid glycoside content in the leaves and reproductive tissues of these *Plantago lanceolata* relative to undamaged controls. Whether damaged or undamaged, the iridoid glycoside content of *P. lanceolata* was highest in the reproductive tissues and lowest in the roots. Although initial concentrations of iridoid glycosides were significantly higher in plants grown under low nutrient conditions than in plants grown under high nutrient conditions, nutrient availability did not alter the phytochemical response of plants to herbivore damage. These results provide additional support for the defensive role of the iridoid glycosides in *Plantago lanceolata* by demonstrating that phytochemical variation is not always an incidental effect of nutrient stress but can be a direct response to damage by herbivores.

Key Words—Phytochemical induction, herbivory, iridoid glycosides, *Plantago lanceolata*.

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INTRODUCTION

Induction of chemical changes resulting from herbivory has been documented in a variety of plant species, including many tree species, a number of crop plants, and some wild or "noneconomic" plant species (see Karban and Baldwin, 1997, for a detailed review). The chemical participants implicated in these induction responses include alkaloids (tomato, tobacco, lupines), proteinase inhibitors (potato), furanocoumarins (wild parsnip), monoterpenes (mints), and phenolics (many species) (Karban and Baldwin, 1997, and references therein). Increasing numbers of empirical studies have shown that phytochemical induction benefits plants by causing reduced herbivore feeding and growth, at least in the short term (e.g., Haukioja and Niemela, 1977; Tallamy, 1985; Edwards et al., 1986; Baldwin, 1988; Gibberd et al., 1988; Zangerl, 1990; Stout and Duffey, 1996; Stout et al., 1998). However, the effects of resource availability (such as light, nutrients, or water) on patterns of phytochemical induction have been more rarely investigated (Hunter and Schultz, 1995; Karban and Baldwin, 1997).

Induced chemical responses may be immediate and short-lived, occurring in as little as 1–4 hr (Dixon et al., 1986; Schultz and Baldwin, 1982; Baldwin, 1988), or may accumulate slowly and persist for one or more years (Faeth, 1986; Haukioja, 1980; Neuvonen and Haukioja, 1984). Depending on the lag time between attack and deployment of the defense, phytochemical induction may either have adverse consequences for the herbivore that initiated the response or influence food preferences and feeding behaviors of herbivores encountering the resource later in the season or even in subsequent years (Karban and Meyers, 1989; Clausen et al., 1991). Thus the timing of induced responses may be important in whether or not, and how, particular herbivores are affected.

Although the effects of nutrient availability on plant allocation to secondary metabolites has been studied in many plant species (e.g., Fajer et al., 1992; Baldwin et al., 1993, Zangerl and Berenbaum, 1995; Hugentobler and Renwick, 1995; Wilkens et al., 1996), few studies have investigated the effect of nutrients on induced responses (Zangerl and Berenbaum, 1995; but see Bryant et al., 1993; Hunter and Schultz, 1995; Stout et al., 1998). The effects of nutrient availability on induced responses (if they occur and what they are) are important for resolving the question of whether the biochemical changes in plant tissues following herbivore damage represent an active response of plants to herbivory, or if these changes are an incidental consequence of defoliation-induced alterations in the carbon–nutrient balance of the plant (Tuomi et al., 1984, 1988, 1990; Haukioja and Neuvonen, 1985; Bryant et al., 1983, 1988, 1993; Hunter and Schultz, 1995). Defoliation may result in the loss of proportionately more nutrients than carbon, thus causing a relative nutrient deficiency in the plant (Chapin, 1980; Tuomi et al., 1984; Bryant et al., 1991), resulting in an increased carbon–nutrient ratio and increased concentrations of carbon-based secondary metabolites (Tuomi et

al., 1984, 1988, 1990; Bryant et al., 1988, 1991; Herms and Mattson, 1992). Thus, if induction is purely an incidental shift in the carbon–nutrient balance due to defoliation, then induced resistance should be weak or nonexistent in nutrient-rich environments. If, however, induced resistance is an active defense against herbivory, then the biochemical response would occur regardless of nutrient availability (Haukioja and Neuvonen, 1985; Haukioja et al., 1985; Tuomi et al., 1988).

Narrow-leaved plantain, *Plantago lanceolata* L., (Plantaginaceae) and the buckeye butterfly, *Junonia coenia* Hübner (Nymphalidae), which uses plantain as one of its major host plants (Bowers, 1984; Scott, 1986), provide a model system for evaluating the phenomenon of phytochemical induction in a common, nonagricultural, herbaceous, plant. Plantain is a short-lived, weedy perennial, which overwinters as a low-lying basal rosette. In the summer, under appropriate conditions of day length and nutrient availability, *P. lanceolata* produces numerous leaves and spiked inflorescences at the end of fibrous stalks (Cavers et al., 1980; Primack and Antonovics, 1982). It is self-incompatible and wind-pollinated. It contains two iridoid glycosides, aucubin and catalpol, which have known toxic and antifeedant effects on generalist herbivores such as the gypsy moth, *Lymantria dispar* (Lymantriidae), and the southern armyworm, *Spodoptera eridania* (Noctuidae) (Bowers and Puttick, 1988; Puttick and Bowers, 1988). However, for certain specialist insects, such as the buckeye butterfly, *Junonia coenia* (Nymphalidae), the iridoid glycosides serve as larval feeding and adult oviposition stimulants (Bowers, 1984; Pereyra and Bowers, 1988).

A series of field experiments on *P. lanceolata* showed that in some cases iridoid glycosides were induced in response to herbivory (Bowers and Stamp, 1993), but in others they were not (Stamp and Bowers, 1996). The reasons for the differences in these results may be due to environmental factors that varied among these experiments (Stamp and Bowers, 1996) or initial constitutive levels of iridoid glycosides at the time of herbivory.

To investigate the role of nutrient availability on induction of iridoid glycosides in *P. lanceolata*, we set up an experiment in which plantain plants, grown under different nutrient availabilities, were exposed or not exposed to herbivores. We were also interested in how above-ground herbivory might affect iridoid glycoside content in leaves as well as roots and reproductive parts. The objectives of this study were to determine: (1) whether damage by herbivores to above-ground plant tissues affected production of iridoid glycosides in *P. lanceolata*; (2) whether phytochemical induction, if it occurred, was an "immediate" (24 hr) or a "longer-term" (28 days) response; (3) whether nutrient availability affected the induction of aucubin and catalpol in *P. lanceolata*; and (4) how allocation of iridoid glycosides to different plant organs (roots, leaves, reproductive parts) varied in response to these treatments.

METHODS AND MATERIALS

Plants used in the experiment were from a single *P. lanceolata* maternal parent. Seeds were germinated in flats in the greenhouse of the University of Colorado EPO Biology Department, Ramaley Building. On March 1, after two weeks of growth, seedlings were transplanted individually into 6-in. round plastic pots containing Term-Lite Metromix soil (a combination of sphagnum peat moss, vermiculite, perlite, pine bark, bark ash, and granite sand). For the duration of the experiment, plants were grown in an environmental chamber with a photoperiod of 16 hr and day–night temperatures of 27°C/25°C, at approximately 70% relative humidity. Plants were randomized within the chamber weekly in order to minimize pseudoreplication effects.

The experiment was a full factorial design with two levels of herbivory [herbivory and no herbivory (= control)], two levels of nutrients (high and low), and two harvest dates (1 day and 28 days after herbivory). There were 10 replicates in each cell for a total of 80 plants. Plants were randomly assigned to treatment groups as follows: first, half the plants (40) were assigned to the herbivory group. These were the plants that were damaged by fifth instars of the buckeye butterfly, *Junonia coenia*, and, as treatment plants they served to determine whether damage by herbivores induced increased production of the antiherbivore compounds, aucubin and catalpol, in *P. lanceolata*. The remaining 40 plants were the no-herbivory group. These undamaged plants served as controls.

In order to determine whether phytochemical induction in *P. lanceolata*, if it occurred, was affected by nutrient availability, plants in both the control and herbivory groups were assigned to either the high-nutrient group or the low-nutrient group. Plants in the high-nutrient group, in addition to daily watering, received 50 ml of Hoagland's solution every other day from the first day of planting. Plants in the low-nutrient group received only plain tap water.

Finally, because the field experiments (Bowers and Stamp, 1993; Stamp and Bowers, 1996) had allowed plants to recover from herbivory for about four weeks, we used 28 days as one time period after which to harvest. However, we were also interested in whether induction occurred quickly, within 24 hr, so we analyzed a second set of plants 24 hr after the herbivores had been removed. The undamaged plants (controls) were harvested at these two times as well, to serve as comparative measures at each stage and control for effects of aging. Thus, there was a total of eight treatments in this experimental design, with 10 replicates in each treatment: (1) herbivory, high nutrient, 1-day analysis; (2) herbivory, high nutrient, 28-day analysis; (3) herbivory, low nutrient, 1-day analysis; (4) herbivory, low nutrient, 28-day analysis; and four parallel groups, with no herbivory but the same set of nutrient and harvest dates, that served as undamaged controls.

On April 6, when plants had just begun to flower and had been growing

in the experimental chamber for five weeks, three newly molted fifth instars of *Junonia coenia* were placed on each of the 40 plants assigned to the herbivory treatment. In order to keep the caterpillars from wandering off the plants, all plants were fitted with mesh bags held up from within by a 14-in. bamboo pole and secured around the outside of the pot with a rubber band. Plants in the control (no herbivory) group were likewise fitted in order to minimize possible effects of altered light availability or stress from handling. Caterpillars remained on the plants for 72 hr. At the termination of the damage treatment, all mesh bags and all caterpillars were removed from the plants.

Twenty-four hours after termination of the damage treatment, plants in the 1-day analysis group were harvested. Plants were separated into roots (rinsed free of soil), shoots, and reproductive parts; placed in labeled paper bags; and oven dried at 50°C to a constant dry weight. Plants in the 28-day analysis group remained in the growth chamber and continued to receive nutrients and/or water for an additional four weeks. At the designated time, the 28-day-analysis plants were harvested and dried in the same manner as described above.

After weighing, the dried samples were ground to a homogeneous powder for chemical analysis. Iridoid glycoside content of each sample was analyzed by gas chromatography (Fajer et al., 1992; Bowers and Stamp, 1993). Twenty-five milligrams of each sample were extracted overnight in 95% methanol, evaporated to dryness, and the residue taken up in water and then partitioned with ether. Gas chromatograph analyses were performed on the trimethylsilylated derivatives. Phenyl-β-D-glucose was used as an internal standard.

RESULTS

Pearson correlation coefficients showed that at the 1-day harvest, leaf and scape biomass were significantly correlated (Table 1), while at the 28-day harvest, leaf, root, and scape biomass were significantly correlated (Table 1), indicating that multivariate analysis of variance (MANOVA) was appropriate. In addition, the concentrations of both aucubin and catalpol were highly correlated in roots, leaves, and reproductive stalks (Table 2), again indicating use of MANOVA. Statistical analyses were performed using the GLM program of the SAS or Systat statistical packages, followed by univariate ANOVA where appropriate. All ratios were arcsine square-root transformed prior to analysis. We used Bonferroni corrections for probability values.

Although the amount of tissue eaten by the *J. coenia* larvae was not specifically quantified, a visual estimate of the damage revealed that individual plants subjected to the herbivory treatment each suffered 15–25% tissue loss over the 72-hr period. MANOVA of the biomass of *P. lanceolata* plants (root weight, leaf weight, reproductive weight) one day after herbivory revealed that there was a

TABLE 1. PEARSON CORRELATION COEFFICIENTS (ABOVE) AND PROBABILITY VALUES (BELOW) FOR BIOMASS OF *Plantago Lanceolata*

	Root weight	Leaf weight	Scape weight
One day postherbivory			
Root weight	1.000		
Leaf weight	0.026	1.000	
	NS[a]		
Scape weight	0.362	0.798	1.000
	NS	<0.001	
28 days postherbivory			
Root weight	1.000		
Leaf weight	0.709	1.000	
	<0.001		
Scape weight	0.477	0.681	1.000
	<0.002	<0.001	

[a]NS = not significant.

significant effect of nutrients on biomass, but there was no significant effect of herbivory and no significant interaction of nutrients and herbivory (Figure 1, Table 3). Although a univariate analysis showed that herbivory reduced leaf biomass ($P = 0.022$), this was not significant with a Bonferroni correction. By 28 days after herbivory, plants grown in the high-nutrient treatment were significantly larger than those grown in the low-herbivory treatment (Figure 1, Table 3), and plants exposed to herbivores were significantly smaller than those not exposed to herbivores (Figure 1, Table 3). There was no interaction between nutrients and herbivory (Table 3).

One day after herbivory, analyses of iridoid glycosides showed that there was no effect of herbivory on iridoid glycosides in leaves, roots, or scapes (Table 4, Figure 2), providing no evidence of short-term induction of iridoid glycosides. There was no effect of nutrients on iridoid glycosides in leaves or scapes (not significant after Bonferroni corrections, Table 4, Figure 2), but roots grown under high nutrients had lower levels of iridoid glycosides than those grown under low nutrients (Table 4, Figure 2). There were no significant interactions (Table 4).

In contrast, 28 days after herbivory, leaves and scapes of plants exposed to herbivores had higher concentrations of iridoid glycosides than plants that were not exposed to herbivores (Table 4, Figure 2). This was true for both aucubin and catalpol (univariate analysis following MANOVA, leaves—aucubin: $P < 0.002$; catalpol: $P < 0.002$; scapes—aucubin: $P < 0.001$; catalpol: $P < 0.005$), whereas root iridoid glycoside concentrations showed no effect of herbivory (Table 4, Figure 2). These results indicated that iridoid glycosides were induced in response to herbivory in both leaves and scapes, but not in roots.

TABLE 2. PEARSON CORRELATION COEFFICIENTS (ABOVE) AND SIGNIFICANCE VALUES (BELOW) FOR IRIDOID GLYCOSIDE CONTENT OF *Plantago lanceolata*

	Root		Leaf		Scape	
	Aucubin	Catalpol	Aucubin	Catalpol	Aucubin	Catalpol
One day postherbivory						
Root						
Aucubin	1.000					
Catalpol	0.513	1.000				
	<0.001					
Leaf						
Aucubin	0.212	0.110	1.000			
	NS[a]	NS				
Catalpol	0.017	0.024	0.731	1.000		
	NS	NS	<0.001			
Scape						
Aucubin	0.299	0.110	0.634	0.317	1.000	
	NS	NS	<0.001	<0.05		
Catalpol	0.260	0.112	0.615	0.634	0.769	1.000
	NS	NS	<0.001	<0.001	<0.001	
28 days postherbivory						
Root						
Aucubin	1.000					
Catalpol	0.671	1.000				
	<0.001					
Leaf						
Aucubin	0.447	0.110	1.000			
	<0.005	NS				
Catalpol	0.219	0.021	0.580	1.000		
	NS	NS	<0.001			
Scape						
Aucubin	−0.031	−0.231	0.464	0.376	1.000	
	NS	NS	<0.005	<0.05		
Catalpol	0.101	0.066	0.405	0.678	0.555	1.000
	NS	NS	<0.01	<0.001	<0.001	

[a]NS = not significant.

Twenty-eight days after herbivory, there was no effect of nutrients on iridoid glycoside concentrations of leaves or scapes (Table 4, Figure 2). However, roots grown under low nutrients had higher levels of iridoid glycosides than those grown under high nutrients (Table 4, Figure 2). Univariate analysis of the effect on roots showed that this effect was due to an increase in aucubin (aucubin: $P < 0.001$; catalpol: NS after Bonferroni correction).

However, because we were interested in the effect of nutrient treatment on induction of iridoid glycosides, it was not the main effect of nutrients that was

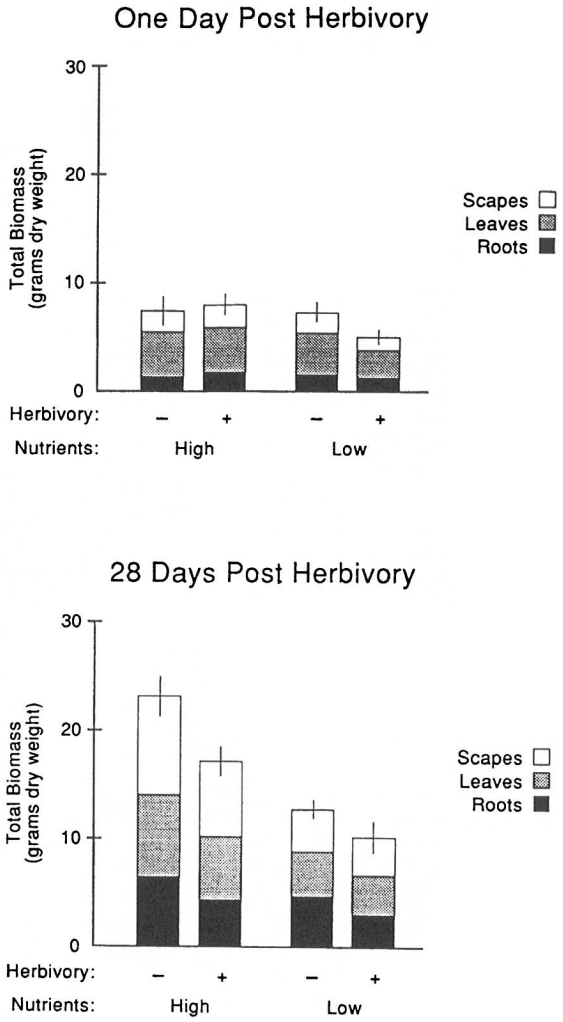

FIG. 1. Biomass (roots, leaves, and reproductive stalks) of *Plantago lanceolata* plants harvested one day after damage by herbivores (top) and 28 days after damage by herbivores (bottom).

of interest, but rather the interaction of nutrients with herbivory. As it turned out, multivariate analysis of variance revealed that in no case was there a significant interaction of nutrients and herbivory (Table 4). Therefore, nutrient availability did not affect the biochemical response of *P. lanceolata* plants to damage by herbivores.

TABLE 3. RESULTS OF MANOVA OF *Plantago lanceolata* BIOMASS[a]

Source of variation	One day postherbivory			28 days postherbivory		
	F	*df*	*P*	*F*	*df*	*P*
Nutrients	6.166	3, 34	0.002	48.789	3, 35	<0.001
Herbivory	1.925	3, 34	0.144	8.731	3, 35	<0.001
Nutrients × Herbivory	1.955	3, 34	0.139	1.816	3, 35	0.163

[a]There were three dependent variables: leaf biomass, scape biomass, and root biomass.

TABLE 4. RESULTS OF MANOVA OF IRIDOID GLYCOSIDE CONCENTRATIONS OF *Plantago lanceolata*[a]

Source of variation	One day postherbivory			28 days postherbivory		
	F	*df*	*P*	*F*	*df*	*P*
Leaves						
Nutrients	2.011	2, 34	0.049	3.130	2, 35	0.056
Herbivory	0.719	2, 34	0.719	7.364	2, 35	0.002
Nutrients × herbivory	1.259	2, 34	0.297	0.065	2, 35	0.937
Scapes						
Nutrients	5.303	2, 34	0.010	0.908	2, 35	0.413
Herbivory	1.473	2, 34	0.244	7.674	2, 35	0.002
Nutrients × herbivory	0.208	2, 34	0.814	0.108	2, 35	0.898
Roots						
Nutrients	8.015	2, 34	0.001	6.786	2, 35	0.003
Herbivory	1.392	2, 34	0.262	3.604	2, 35	0.038
Nutrients × herbivory	0.158	2, 34	0.854	2.687	2, 35	0.082

[a]For each analysis (leaves, scapes, roots) there were two dependent variables: aucubin concentration and catalpol concentration.

DISCUSSION

These results show that iridoid glycosides in *Plantago lanceolata* are induced in response to damage by lepidopteran herbivores. Phytochemical induction did not occur immediately but was evident 28 days after damage by *J. coenia* caterpillars. While nutrient limitation also caused an increase in iridoid glycoside concentration, the availability of nutrients did not have a significant influence on the phytochemical response of plants to herbivore damage. Thus, whether plants are grown under high-nutrient conditions or under low-nutrient conditions, the phytochemical response of *P. lanceolata* to damage by larvae of the buckeye butterfly was the same. This fact lends support to the theory that induced resistance

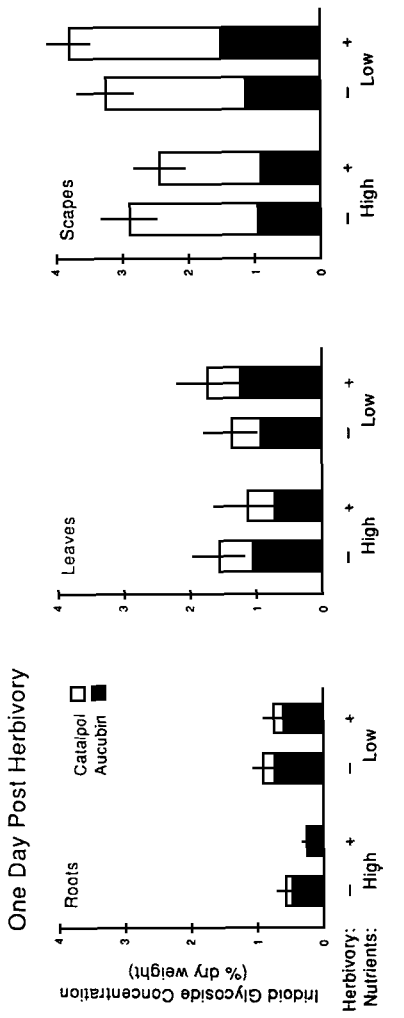

FIG. 2. Iridoid glycoside concentration (aucubin and catalpol as percent dry weight) in the roots, leaves, and reproductive stalks of *Plantago lanceolata* plants harvested one day after damage by herbivores (top) and 28 days after damage by herbivores (bottom).

is indeed an active defense against herbivory and not merely an incidental consequence of defoliation-induced nutrient stress (Haukioja and Neuvonen, 1985; Haukioja et al., 1985; Hunter and Schultz, 1995; see Karban and Baldwin, 1997).

In this study, the increase in iridoid glycoside concentration following herbivore damage was shown to occur in the leaves and reproductive stalks but not in the roots. This pattern is in accordance with the optimal defense theory (McKey, 1974, 1979; Rhoades, 1979; Bazzaz et al., 1987), which predicts that plants should allocate more of their defensive budget to those tissues that are more susceptible to herbivory and more valuable in terms of fitness than less vulnerable, easily replaced tissues. While *J. coenia* caterpillars were observed feeding on both the leaves and the reproductive tissues, they did not directly damage the root tissues. It is possible that if the roots were subject to damage by subterranean herbivores they, too, might exhibit an increase in defensive chemistry.

The timing of the induction response is a critical element in predicting its effectiveness in terms of plant (and herbivore) population dynamics. Depending on the lag time between herbivore attack and deployment of the defense, phytochemical induction may either have adverse consequences for the herbivore that caused the response or it may influence the food preferences, behavior, and oviposition choices of subsequent generations. Although in the case of *P. lanceolata* induction did not occur within 24 hr, it was short-term relative to the growing season and to the life of the plant, but long-term relative to the life of most insect herbivores. Such increases in iridoid glycoside content of leaves and reproductive stalks resulting from herbivore damage may have both positive and negative consequences to the plant. While higher levels of defensive chemistry can protect plant tissues from generalist herbivores (Bowers and Puttick, 1988, 1989), they can, at the same time, render the plant more palatable to adapted specialists (Bowers and Puttick, 1989) since specialists often use secondary compounds as feeding or oviposition cues.

Damage by herbivores also had a significant negative effect on the biomass of plants subjected to herbivory. These results are in contrast to similar studies of the effects of herbivory on *P. lanceolata* (Bowers and Stamp, 1993; Stamp and Bowers, 1996), which showed that this species has the ability to compensate for biomass lost to herbivory. The differences in the results of these studies may be due to several factors. One of the most compelling is that these plants were grown in a growth chamber. This might exemplify plants grown under low light conditions in which sufficient carbon might not be available to allow plants to compensate for lost tissue (e.g., Maschinski and Whitham, 1989; Karban and Baldwin, 1997).

Depending on the lag time between the initial attack and the response of the plant, and depending on how long the induced levels of phytochemicals persist, the effects of induction may be quite different. For example, in this experiment

the induction of iridoid glycosides was evident 28 days after the herbivore damage. After this amount of time, it could be the early instars of the next generation of buckeyes that would be exposed to the increased iridoid glycoside level. Since early instars are typically more susceptible to phytochemicals than later instars (Scriber and Slansky, 1981), this time course of induction could be more important for future generations rather than the generation that did the damage. However, we did not look at plants at a time intermediate between 1 and 28 days, so we were unable to determine the exact time course of induction of iridoids in *P. lanceolata*. This is clearly something that needs to be addressed in the future for different species of herbivores.

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PREFERENCE FOR ACYANOGENIC WHITE CLOVER (*Trifolium repens*) IN THE VOLE *Arvicola terrestris*: I. EXPERIMENTS WITH TWO VARIETIES

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Abstract—We report experimental results showing that, under both laboratory conditions as well as in outdoor enclosures, the fossorial vole *Arvicola terrestris* preferentially feeds on acyanogenic white clover (*Trifolium repens*) when offered the choice between two varieties (Ladino and Aran) differing highly in their content in cyanogenic glycosides. We also observed that the voles adapted their diet and reduced their relative consumption of the cyanogenic variety during experiments conducted for two to three weeks in outdoor enclosures as compared to shorter tests conducted for 48 hr in laboratory cages. In addition, we report a similar preference for the acyanogenic Ladino variety for the slugs *Arion ater* and *A. subfuscus*.

Key Words—Cyanogenesis, voles, *Arvicola terrestris*, white clover, *Trifolium repens*, Ladino, Aran, *Arion ater*, *Arion subfuscus*.

INTRODUCTION

Small mammals (voles, lemmings) have long been known to exhibit "population cycles," i.e., strong fluctuations in numbers recurring at three- to four-year intervals (Krebs and Myers, 1974). The origin of these fluctuations is still considered to be an enigma (Lidicker, 1988), and more than 20 explanatory hypotheses have been proposed to account for this puzzling phenomenon (Batzli, 1992). Among

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these hypotheses, the roles of food quality and of plant secondary compounds often have been evoked.

Among the many classes of plant secondary compounds that can affect herbivores, cyanogenic glycosides are of particular significance because of their acute toxicity (Seigler, 1991). Cyanogenesis (the ability of plants to produce and store cyanogenic glycosides that release HCN when hydrolyzed by a specific β-glycosidase) has been reported in many plant species (Hegnauer, 1986; Seigler, 1991). In *Trifolium repens* (white clover), cyanogenic glycosides are concentrated in the leaves and do not occur in the roots and the stems. The enzyme (linamarase EC 3.2.1.21) is located in the cell walls and the substrate (linamarin and lotaustralin) in the cells (Kakes, 1985; Kakes and Eeltink, 1985).

The genetic basis of cyanogenesis in *T. repens* and *Lotus corniculatus* is well known (Hughes, 1981), and a widespread polymorphism for cyanogenesis has been reported to occur in natural populations (reviewed in Jones, 1988). In addition, a temporal and intraplant variability in the expression of cyanogenesis has also been found in *T. repens* (Ramnani and Jones, 1985; Till-Bottraud et al., 1988).

The maintenance of the polymorphism for cyanogenesis in natural populations remains poorly understood with many observations in contradiction with Daday's hypothesis (1954) that temperature is a sufficient factor to explain this phenomenon (reviewed in Jones, 1988, 1998). As an alternative hypothesis, Ellis et al. (1977) proposed that this polymorphism could be maintained by herbivorous mollusks and suggested that cyanogenic morphs were few when slugs and snails were rare and common when mollusks were abundant. The defensive function of cyanogenesis has been documented in many studies with ample evidence that many invertebrate herbivores, mostly mollusks and insects, were deterred from feeding on cyanogenic plants (Jones, 1962, 1966; Crawford-Sidebotham, 1972). However, a critical review of the evidence indicates that a preference for acyanogenic plants has been shown for only 53% of the herbivore species tested and that many studies in which such a preference has been allegedly found lack an appropriate statistical treatment (Hruska, 1988).

Apart from experiments by Jones (1962, 1966), who has tested two individual voles (*Microtus agrestis*), few studies involving mammals have been conducted. Differential grazing between cyanogenic and acyanogenic plants has been suggested from field studies in deer and rabbits (Corkill, 1952; Cooper-Driver and Swain, 1976), but no selective eating could be detected in sheep (Corkill, 1952). Sherbrooke (1976) reports field and laboratory evidence for differential consumption of cyanogenic jojoba seeds by various desert rodents, while Compton et al. (1983) observed in a field study that cyanogenic phenotypes of *L. corniculatus* were more frequent in areas of high lemming (*Lemmus lemmus*) densities in Norway and suggested a link between the dynamics of fluctuating rodent populations and changes in the proportions of cyanogenic *Lotus* plants.

In order to investigate this hypothesis, we took advantage of the fact that fossorial *Arvicola terrestris* is a highly selective and destructive herbivore that feeds preferentially on *T. repens* under both field and experimental conditions (Kopp, 1988, 1993). Different from invertebrates, whose major impact might be restricted to early developmental plant stages (e.g., on seedlings or germinating plants), voles are likely to destroy well-established plants over wide areas. In addition, populations of this rodent show strong fluctuations in numbers with periods of high and low densities recurring every five to eight years (Saucy, 1988, 1994). Furthermore, during a field study encompassing the duration of such a population cycle, corresponding cyclic changes in the abundance of *T. repens* have been recorded by Kopp (1993), who suggested that cyanogenesis could be involved in this process.

In this paper, we report results of tests conducted in the laboratory with two agronomic *T. repens* varieties (Ladino and Aran) differing strongly in their cyanogenic properties. Our goals were to determine whether voles are able to discriminate between cyanogenic and acyanogenic plants, and whether they are deterred from feeding on the former. In addition, we conducted complementary experiments with the same cultivars and slugs of the genus *Arion*.

METHODS AND MATERIALS

In order to investigate the ability of fossorial *A. terrestris* to distinguish between cyanogenic (CN^+) and acyanogenic (CN^-) plants, we performed two series of experiments, one conducted in cages and the other in outdoor enclosures. In both series of tests, animals were offered a choice between two white clover varieties: the weakly cyanogenic variety Ladino (L; origin: plain of the Po river, Lodi, Italy) and the strongly cyanogenic variety Aran (A; Oak Park Centre, Carlow, Ireland). These varieties are known from the literature for their extreme quantitative differences in cyanogenic glycosides, the ratio of Ladino/Aran for cyanogenic glycosides being approximately 1/100 (cyanide content: 26 and 2405 mg HCN/kg of dry matter, respectively) (Lehmann et al., 1991), while the percentage of cyanogenic individual plants in these varieties is less than 2% in Ladino and higher than 98% in Aran (Caradus, 1986; Williams, 1987). Both Ladino and Aran are large-leafed varieties and are phenologically very similar.

Plants were grown from seeds sown in the spring in rectangular flowerpots (cage experiments) or in square outdoor tanks (outdoor enclosure experiments) and voles were allowed to feed on live plants. In both experiments, the containers were divided into four subunits. Seeds of each of the two varieties were sown in two of these subunits according to a 2×2 alternate design, with plants of the same variety sown in opposite positions (i.e., in the top right and bottom left subunits and reciprocally). In addition, the disposition of the CN^+/CN^- varieties was random-

ized among pots. Experiments were started when the plants had grown in a regular and uniform canopy. Because these varieties are large-leafed and phenologically very similar, it was impossible (at least for us) to detect any pattern indicating the sowing design. It is therefore reasonable to assume that the voles could not rely on visual cues when they selected to feed in a particular plot.

Cage Tests in the Laboratory. For these experiments, we used the Ladino California cultivar, which is commonly included in many seed mixtures, and the cyanogenic Aran variety. In April 1994, plants were sown in heat-sterilized compost and grown in rectangular 40 × 30 × 10-cm flowerpots for about three months in a greenhouse. Plants were mowed after three weeks in order to stimulate vegetative growth. To avoid having the voles dig into the flowerpots during the tests and destroy the whole experimental set up, pots were covered at sowing time with a 10 × 10-mm wire netting (diameter 0.5 mm) and plants were allowed to grow in the interstices. Physical separations between the four sowing subunits of each flowerpot allowed us to accurately harvest each of the two white clover varieties.

The cage tests were performed between July 29, 1994, and August 19, 1994. Field-caught voles kept in captivity for four to five weeks were placed in 40 × 50 × 40-cm wire cages in which the experimental flowerpots were lying on the floor. The 5-cm-wide spaces between the flowerpots and the opaque walls of the cages were covered with a wooden lid, which created a system of runways mimicking a burrow system. Four openings in the lids (one on each side of the cage) allowed the voles to reach the plants. Voles readily adopted this set-up and built their nests below the lids. Preliminary tests showed that the voles consumed approximately 50% of the offered food within two days, and the tests were therefore run for 48 hr. No water or alternative food was provided.

Data Analysis. By offering living plants to the animals, there was no nondestructive way of getting direct estimates of the quantity of each variety offered to the voles before the tests and, therefore, of getting estimates of the quantity of food actually eaten in each particular test. For this reason, we used an indirect approach. We decided to compare estimates of the remaining dry matter of both varieties at the end of the tests against a series of control pots. In this approach, preferences can be expressed as ratios between the acyanogenic and cyanogenic varieties, i.e., Ladino over Aran (L/A or CN^-/CN^+) with smaller or larger ratios in the experimental setting than in the control tests indicating a preference for the acyanogenic or the cyanogenic variety, respectively. Statistically significant differences between control and experimental tests were detected with the nonparametric Mann-Whitney test, and one-tailed comparisons were performed because we expected a preference for the acyanogenic variety. Therefore, at the end of the tests, the vegetation remaining in experimental pots was cut (separately for each variety) and dried overnight in an oven at 55°C. Control pots were treated in a similar way. Cyanide contents of both varieties were estimated from preserved

samples from the control pots. These analyses were performed in the laboratory of the Swiss Federal Station for Animal Production, CH-1725 Posieux, by the distillation method described by Pulss (1962).

Tests in Outdoor Enclosures. In addition to the laboratory cage tests, complementary experiments were conducted in outdoor enclosures in which voles were allowed to dig underground burrows, to feed, and to behave in their usual way. Sixteen polyester tanks ($2 \times 2 \times 0.5$ m) arranged in a 4×4 design were built and placed on the ground at the perimeter of the university campus. They were filled with a bed of 35 cm of earth from a neighboring natural grassland. The tanks were covered with "vole-proof" lids to restrain the animals from escaping, as well as to protect them from predators. Small holes (1 cm diam.) in the bottom of the tanks allowed for water drainage.

In June 1994, two varieties of *Trifolium repens* (Ladino and Aran) were sown according to the 2×2 alternate design described earlier in four contiguous 0.8×0.8-m squares located in the center of the tanks. In order to minimize edge effects along the walls of the tanks, the remaining 20-cm-wide strip was sown with rye grass (*Lolium perenne;* a grass well accepted by fossorial *A. terrestris* when dicots are absent) (Kopp, 1993). In this case, however, Ladino California was replaced by the even less cyanogenic Ladino Espanso (Lodi, Italy; <10 mg HCN/kg dry matter) (Lehmann et al., 1991), after it was realized that the Californian cultivar had been imperfectly controlled by the breeder for its content in cyanoglucosides, as compared to the original Italian variety.

In July 1994, three weeks before the beginning of the experiments, 20 voles (10 males and 10 females) were paired and became accustomed to each other in laboratory cages. In August, they were released in 10 enclosures assigned at random among the 16 tanks. The six remaining tanks were used as control units. Experiments were run for periods varying between 11 and 29 days (average: 18 $\pm$ 2 days) depending on the rate at which the voles had consumed approximately half of the available vegetation.

Data were analyzed in a similar way as in the cage experiments and are expressed as ratios of dry matter for both varieties (CN^-/CN^+ ratios). In addition to these measures, we also recorded at two-day intervals the above-ground signs of vole activity in the enclosures (vole hills, holes, feeding signs, etc.). Preserved samples of *T. repens* were also analyzed for their cynanide content.

Tests with Slugs. In addition, we conducted tests with slugs of the genus *Arion* (*A. ater* and *A. subfuscus*) in order to determine whether these invertebrates, which had been widely used in early demonstrations of the role of cyanogenesis (Crawford-Sidebotham, 1972; Jones, 1988), would support our observations with voles. Plants were grown from seeds in $25 \times 25 \times 12$-cm pots in spring 1996, either in pure Ladino Espanso or Aran stands or in mixtures (according to a 2×2 alternate design in the latter case) with eight replications of each of the three treatments. After germination, the number of plants per pot was adjusted

to 20 and the plants were allowed to grow for 45 days in the greenhouse. The pots were covered with gauze to protect the plants from herbivores. Three slugs were introduced for five days in each pot (until approximately half of the leaves had been consumed). The pots were regularly watered to keep the slugs alive and active and remained covered to prevent the mollusks from escaping. At the beginning and at the end of the experiments, the number of white clover leaflets were counted and the slugs were weighed.

RESULTS

Tests in Laboratory Cages. The results from tests in laboratory cages indicate that the voles left quantitatively less or ate more of the acyanogenic Ladino variety compared to the cyanogenic Aran (Table 1). On average, the L/A ratio dropped from 0.94 ± 0.08 (mean $\pm$ SD; $N = 11$) in control pots to 0.68 ± 0.23 in experimental trials ($N = 13$ animals tested). Therefore, a clear bias of the CN^-/CN^+ ratio was observed, indicating that the voles ate more of the Ladino variety, the acyanogenic form. The average difference in ratios between control and experimental pots is 0.26 and is significant ($z = -2.926$, $P < 0.01$, Mann-Whitney nonparametric test, one-tailed). The results (Figure 1a) show a scatter of CN^-/CN^+ ratios, ranging between extreme preference for the acyanogenic variety (ratio = 0.23) to lack of discrimination (ratio = 1.0). No indication of a preference for the cyanogenic variety was recorded. The average cyanide content was estimated to be approximately 9 times lower in Ladino California (160 ± 42 mg HCN/kg of dry matter; $N = 8$) than in Aran (1416 ± 120 mg/kg; $N = 10$; Table 1).

Tests in Outdoor Enclosures. In outdoor enclosures, the results were similar and consistent with those gathered in the cage experiments (Table 1). On average, the experiments lasted for 18 days. In this case, the average CN^-/CN^+ ratio was 1.17 ± 0.16 and 0.89 ± 0.24 for control and experimental trials, respectively. Therefore, the voles also showed a bias in favor of acyanogenic white clover in the outdoor enclosures, with an average difference in ratios of 0.28 (very close to the difference of 0.26 in the cage experiment). As in the cage tests, this difference is significant ($z = -3.322$, $P < 0.02$, Mann-Whitney, one-tailed). As a whole, CN^-/CN^+ ratios ranged from 0.5 to 1.3 for experimental trials and from 1.0 to 1.4 for controls (Figure 1b). Eight of the 10 pairs of voles showed a preference for the acyanogenic form, while two pairs did not. However, no pair showed a preference for the cyanogenic form. As a main difference with the cage tests, Ladino Espanso was significantly more productive than Aran in the outdoor enclosures.

The cyanide content was estimated to be 1369 ± 140 mg/kg for Aran ($N = 6$). For the Ladino Espanso variety, three of six samples tested were below the detection level of the method, and the three remaining samples showed a level of 14 ± 3 mg HCN/kg dry matter (Table 1).

TABLE 1. RESULTS OF CHOICE TESTS IN LABORATORY CAGES AND OUTDOOR ENCLOSURES WITH *Arvicola terrestris* AND LADINO (ACYANOGENIC) AND ARAN (CYANOGENIC) VARIETIES OF *Trifolium repens*[a]

Variety	Average dry matter (g ± SD)		Average diff. (C − E) (g)	Average HCN content [mg/kg (mean ± SD)]	Estimated HCN intake (mg/indiv/day)
	Control group	Experimental group			
Laboratory cage experiments	(N = 11)	(N = 13)			
Ladino (California)	18.6 ± 4.99	7.9 ± 5.83	10.7	160 ± 42 (N = 8)	0.86
Aran	19.8 ± 5.28	11.0 ± 6.96	8.8	1416 ± 120 (N = 10)	6.23
Ratio L/A	0.94 ± 0.08	0.68 ± 0.23	0.26		
Outdoor enclosure experiments	(N = 6)	(N = 10)		(N = 6)	
Ladino (Espanso)*	219.9 ± 67.0	113.1 ± 29.4	106.8	14 ± 3	0.04
Aran	175.6 ± 36.8	133.7 ± 39.9	41.9	1369 ± 140	1.59
Lolium	451.0 ± 56.4	327.9 ± 70.3	123.1		
Ratio L/A	1.17 ± 0.16	0.89 ± 0.24	0.28		

[a]Average dry matter: average dry matter (g) harvested for each variety at the end of the experiments. Average of 11 and 13 cases in cage experiments and of 6 and 10 experimental units in outdoor tanks for control and experimental groups, respectively. Average diff. (C-E): average difference in harvested dry matter (g) between the control and the experimental groups. Average HCN content: average content in cyanoglucosides for both varieties expressed as mg HCN/kg of DM. Estimated HCN intake: estimates of the daily intake in cyanoglucosides per individual (expressed in equivalents of mg HCN/kg of body mass).

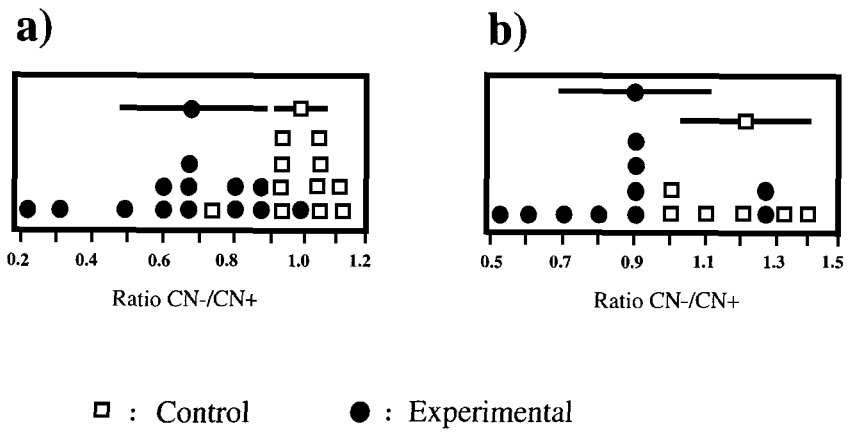

FIG. 1. Detailed results of the tests conducted with the vole *Arvicola terrestris* and the two varieties of *Trifolium repens*. Black dots and white squares indicate results of individual observations for experimental and control trials, respectively, in (a) choice tests conducted in laboratory cages and (b) in outdoor enclosures. Average values with standard deviations are indicated on top of the graphs.

Tests with Slugs. As shown in Table 2, slugs also preferred the acyanogenic Ladino variety. At the beginning of the tests, each of the 20 plants in a pot had produced approximately four leaves or 12 leaflets, corresponding to an average of about 240 leaflets per pot. When the plants were offered in pure stands, the slugs ate significantly more of the Ladino (CN^-) variety (54% or 138 of 256 leaflets on average), as compared to 35% (83 of 233) of the Aran cyanogenic morph (Table 2; $z = -2.896$, $P < 0.01$, one-tailed Mann-Whitney test). Slugs were even more selective when the plants were offered in mixed stands. On average, they ate 71% and 33% of each variety, respectively (Table 2; $z = -3.046$, $P < 0.01$, one-tailed Mann-Whitney test). In addition, it was possible with mixed stands to perform statistical tests for each individual trial, and differences were found in seven of the eight cases (Table 2; $P < 0.01$; χ^2). Figure 2 also shows that the slugs ate significantly more (Figure 2a) and increased more in body mass (Figure 2b) when they were allowed to feed on acyanogenic plants.

DISCUSSION

The results of these experiments with two varieties of *Trifolium repens* (Ladino and Aran) experimentally confirm and extend data from vole and plant species from previous observations reported by Jones (1962, 1966) for *Microtus agrestis* and *Lotus corniculatus*. In contrast with the earlier studies, we tested

TABLE 2. RESULTS OF CHOICE TESTS CONDUCTED IN LABORATORY WITH SLUGS (*Arion* sp.) AND LADINO ESPANSO (ACYANOGENIC) AND ARAN (CYANOGENIC) VARIETIES OF *Trifolium repens*[a]

	Number of leaflets at the beginning		Number of leaflets remaining at the end		Number of leaflets eaten			
	Aran (CN^+) ($N = 7$)	Ladino (CN^-) ($N = 8$)	Aran (CN^+) ($N = 7$)	Ladino (CN^-) ($N = 8$)	Aran (CN^+) ($N = 7$)	Ladino (CN^-) ($N = 8$)	P	Statistical test used
Pure stands								
Average	233.1	256.5	150.0	118.4	83.1	138.1		1-tailed
Range	210–261	234–261	136–167	73–168	47–125	110–182		Mann-
SD	14.8	14.4	11.5	28.5	22.0	23.5	<0.01	Whitney
Mixtures of Ladino and Aran								
Trial 1	144	120	90	35	41	72	<0.01	χ^2
Trial 2	108	114	78	16	25	93	<0.01	χ^2
Trial 3	111	114	75	39	33	72	<0.01	χ^2
Trial 4	108	123	78	42	30	81	<0.01	χ^2
Trial 5	129	129	75	40	51	86	<0.01	χ^2
Trial 6	84	96	68	15	13	78	<0.01	χ^2
Trial 7	111	93	69	46	27	32	>0.05	χ^2
Trial 8	114	126	79	32	26	85	<0.01	χ^2
								1-tailed
Average	113.6	114.4	76.5	33.1	30.8	74.9		Mann-
SD	16.3	12.5	6.4	10.9	11.4	18.7	<0.01	Whitney

[a]Global results of the experiments with 20 plants sown in pure stands and detailed results of the eight trials for tests with plants sown in mixtures of the two varieties (10 Aran and 10 Ladino plants). The number of leaflets present at the beginning and at the end of the tests are given for the two varieties. The number of leaflets eaten has been calculated under the assumption that the plants did not produce new leaves during the course of the experiments. Number of eaten leaflets = number at the beginning − number at the end − number wasted.

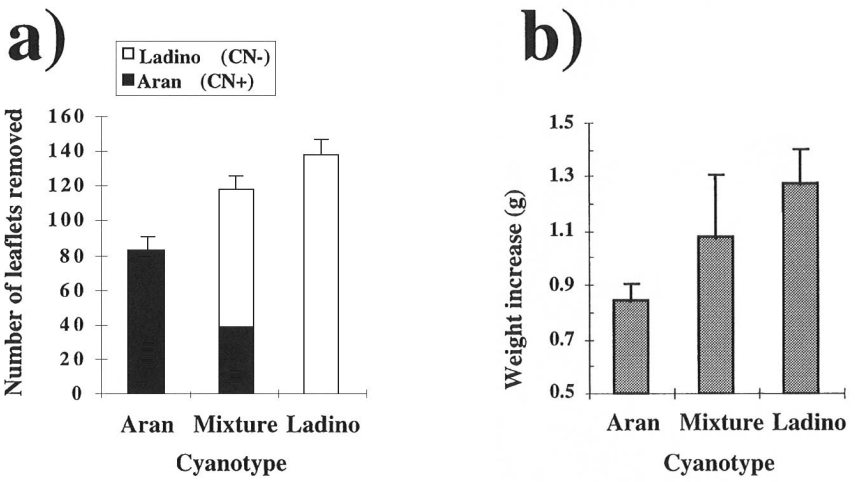

FIG. 2. Results of the choice tests conducted with slugs (*Arion* sp.) and the two varieties of *Trifolium repens*. (a) Average slug consumption in pure and mixed stands, (b) average individual change in slug body mass. Vertical bars indicate standard errors.

a larger number of individual voles (total = 33 in 23 experimental units, as compared to two individuals in the same pit, or one experimental unit for *M. agrestis*). Furthermore, our results have been replicated in two different experiments, one of which attempted to mimic natural field conditions. Our results indicate that fossorial *A. terrestris* are able to distinguish between two varieties of white clover, presumably on the basis of their differences in cyanogenic glycoside content. Complementary experiments with slugs of the genus *Arion* also confirm the well-established results obtained by various authors more than 20 years ago (Jones, 1988, 1998).

Comparisons between control and experimental tests, however, also show that voles ate substantial amounts of the cyanogenic morph, a result that has been reported in many studies both in mammals and in invertebrates (Jones, 1998). If we estimate the food consumed as the difference $(C - E)$ between control (C) and experimental trials (E) for both forms and the relative proportion as $(C-E)/C$, voles ate on average 57.6% and 44.4% of the acyanogenic Ladino and the cyanogenic Aran standing crops, respectively. It is also worth mentioning that in several cases, we noted that the voles only ate the leaves of the plants, leaving the stems untouched. This always affected the acyanogenic Ladino variety (in *T. repens* cyanogenic glycosides are concentrated in the leaves). Therefore, the differences in consumption reported above may have been partly underestimated.

During our two-day laboratory cage experiments, the voles (weighing 70.8 ± 2.3 g on average at the beginning of the experiment) ate on average, 19.8 g

of dry matter, or 66 g of fresh food per day. This amount corresponds approximately to 93% of their body mass, which is in the range of what is known for the physiology of this animal (Grenot et al., 1984; Kopp, 1993), especially if we take into account that part of the food was wasted. We estimated that the voles consumed on average 7.1 mg of cyanide per day which corresponds to a dose of 101 mg HCN/day/kg body mass. No animal died during the following days or weeks, and they maintained constant body weights during the experiment (average weight change = 0.1 ± 2.0 g, ranging from −3 to +4 g). By comparison, the lethal dose of KCN when taken orally in a single dose is 3–5 mg/kg in humans (Seigler, 1991). The LD_{50} of KCN in mice is 2–6 mg/kg (intraperitoneal injections) and approximately three times higher in the rat (Seigler, 1991; Speijers, 1993). Although our animals did not ingest pure cyanide, these results indicate that the absorbed doses are, by all standards, high for mammals. It should be remembered, however, that mammals can detoxify large amounts of cyanogenic glycosides when they are ingested in small quantities over long periods of time (Jones, 1998).

Applying the same line of reasoning for outdoor experiments, we estimated that each pair of voles consumed on average 106.8 g dry matter of the Ladino variety (approximately 48.6% of the available acyanogenic biomass) and only 41.9 g dry matter of the Aran variety (23.9% of the cyanogenic biomass). Thus, they ate approximately 2.5 times more of the nontoxic form of white clover. They also consumed 123 g dry matter of *Lolium perenne* (27.3% of the grass standing crop). We estimated the vole individual daily consumption to be approximately 63 g of fresh food per day. Cyanogenic plants accounted for only 15.4% of the total ingested biomass, as compared with 44% in the cage experiments. On average, the voles ingested of 1.6 mg HCN/individual/day, which corresponds to approximately 25 mg HCN/kg body mass.

In contrast to the results of the cage experiments, in this case voles were in poor condition at the end of the experimental period. Interestingly, one individual was not recaptured and probably died in the tank, while two voles died in the animal house within a few days after having been removed from the enclosures. Moreover, 14 of the 19 individuals lost weight (between 12 and 23 g for six of them). The average change in body mass was −6.4 ± 2.0 g and was significant statistically ($t = -3.305$, $P < 0.01$, two-tailed t test, $df = 18$). Because it may be argued that animals from the same tanks do not correspond to independent units, we made the same calculations by using the average difference in body mass for the pairs in each enclosure with similar results (average difference: −6.3 ± 2.6 g; extremes: −20.5 and +3 g; $t = -2.561$, $P < 0.05$; two tailed t test; $df = 9$).

The results of the experiment in outdoor enclosures suggest that, apart from being in poor condition, fossorial *A. terrestris* significantly reduced their total consumption of the toxic morph from 101 mg HCN/day/kg during the 48-hr tests to a level of approximately 25 mg HCN/day/kg during experiments lasting

for 18 days. Therefore, voles ingested amounts of cyanide that were in the upper range of what is still considered as sustainable for many mammals (Jones, 1998), but can cause serious reproductive trouble in sheep and cattle (Lehmann et al., 1991; Gutzwiller, 1993). This reduction in cyanogenic white clover consumption indicates that voles might have progressively adapted their diet in reducing its content in cyanoglycosides, while the important weight losses recorded during the study, as well as the deaths, suggest that voles may have been intoxicated by high amounts of cyanide intake, a point that would be worth investigating further.

Both laboratory cage and outdoor enclosure experiments indicate that when given the choice, fossorial *Arvicola terrestris* are able to discriminate between cyanogenic and acyanogenic forms of white clover and prefer the latter. It could be objected that in our experiments we cannot exclude that the voles preferred the Ladino morph for reasons unrelated to its low cyanic content. In order to exclude this possibility, we should have worked with different selected cyanotypes. For logistic and practical reasons and because the amount of material needed for the tests was so large (tens of square meters) and exceeded by far our cultivation and breeding capacities, we decided to work with two varieties differing strongly in their cyanide contents. As a complementary approach, we have extended our experiments to six varieties using a different experimental approach. The results, which will be reported in a future publication (Viette et al. in preparation), fully confirm that voles prefer the less cyanogenic cultivar. The fact that slugs, which are unrelated organisms and which have long been shown to be very sensitive to cyanogenic glycosides, also preferred the acyanogenic variety, can be considered as indirect evidence in support of our interpretation that the preference recorded for the Ladino cultivar is mainly related to its low content in cyanogenic glycosides.

In conclusion, in this paper we confirm and extend to the vole *Arvicola terrestris* and to the plant *Trifolium repens* early observations of Jones (1962, 1966) on *Microtus agrestis* and *Lotus corniculatus*. We show that fossorial *Arvicola terrestris* feed preferentially on acyanogenic plants when given the choice. Our observations also suggest that these voles seem able to adapt their diet and cyanide intake to sustainable levels, which are similar to those reported for several other mammal species.

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EFFECTS OF CYANOGENESIS POLYMORPHISM IN *Turnera ulmifolia* ON *Euptoieta hegesia* AND POTENTIAL *Anolis* PREDATORS

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Abstract—We examine the effects of the cyanogenesis polymorphism in *Turnera ulmifolia* on larvae, pupae, and adults of *Euptoieta hegesia*, the most damaging herbivore of *T. ulmifolia* in terms of tissue loss per unit time. We provide evidence that female *E. hegesia* do not show preference for host plants on the basis of their cyanogenesis level but do prefer *T. ulmifolia* over equally cyanogenic, closely related secondary host-plant species (*Passiflora* sp.). Similarly, cyanogenesis in *T. ulmifolia* has little effect on the food preference, growth, or development of the larvae. The potential host range of *E. hegesia* is limited, even within the genus *Turnera*, but this does not appear to be due to host-plant cyanogenesis. Pupae suffer very high mortality levels in the wild that are not associated with host-plant cyanogenesis, although our studies indicate that larvae are capable of sequestering cyanogenic glycosides from their host plants and possibly of synthesizing these or similar compounds. We provide evidence that the presence of sequestered cyanogenic compounds in the larvae protects them from terrestrial-based predators such as *Anolis* lizards

Key Words—*Turnera ulmifolia*, *Euptoieta hegesia*, cyanogenesis, *Anolis sagrei*, tritrophic interactions, host range, sequestration, growth, development, oviposition.

INTRODUCTION

A wide variety of plant chemical defenses has been shown to act as deterrents to colonization or herbivory by generalist herbivores (Bernays and Chapman,

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1987; Jones, 1988; Nahrstedt, 1988; Zangerl and Berenbaum, 1993). However, these defenses may also act as oviposition or gustatory acceptability cues or be coopted for other purposes (e.g., the defense of the herbivore against its own predators or parasites) by specialist herbivores that are capable of recognizing, detoxifying, and sequestering the chemical defense, or are otherwise immune to the defense (Ehrlich and Raven, 1964; Ellis et al., 1977; Woodhead and Bernays, 1978; Brattsten et al., 1983; Compton and Jones, 1985; Bernays and Chapman, 1987; but see Dritschilo et al., 1979). Therefore, it is important in any assessment of potential plant–herbivore coevolution that the effects the plant chemical defenses have on the herbivore be assessed.

Plant species that are genetically variable for defensive traits are especially useful systems for such investigations because they allow direct assessment of the usefulness and efficacy of the defense against any or all of the suite of herbivores that prey upon the plant (Jones, 1971, 1988; Burgess and Ennos, 1987). *Turnera ulmifolia* L. (Turneraceae) exhibits extensive genetic variation for a defensive trait, cyanogenesis, within and between populations in Jamaica (Schappert and Shore, 1995). Damage to plant tissues of this species results in the liberation of HCN and a ketone when a cyclopentenoid cyanogenic glycoside comes in contact and is hydrolyzed by a β-glycosidase enzyme (Tober and Conn, 1985; Seigler, 1991). Both end products have been implicated as being toxic and/or acting as deterrents to herbivory (Compton and Jones, 1985; Peterson, 1986; Jones, 1988; Nahrstedt, 1988; Spencer, 1988; Seigler, 1991; but see Scriber, 1978, and Woodhead and Bernays, 1978). The disruption of tissues is necessary before cyanogenesis can occur but, depending on the quantity of the precursors present, the trait can act quickly when tissue disruption does occur (Seigler, 1991; Berenbaum and Seigler, 1992).

Euptoieta hegesia Cramer (Lepidoptera: Nymphalidae) uses *Turnera ulmifolia* as its primary host plant on the island of Jamaica (Schappert and Shore, 1998; and see below) but is also known to use a variety of cyanogenic *Passiflora* spp., especially *P. foetida* L. and *P. suberosa* L. (Passifloraceae), as secondary host plants (Schappert and Shore, 1998). Neither of these species is known to vary for cyanogenesis. This herbivore is the most damaging (in terms of tissue loss per unit time) of the herbivores commonly found on *T. ulmifolia* in Jamaica (Schappert, 1997; Schappert and Shore, in press). Larvae of this species are distributed nonrandomly among *T. ulmifolia* hosts in Jamaica (Schappert and Shore, 1998), and eggs and larvae tend to be more common on less cyanogenic plants (Schappert, 1997; Schappert and Shore, in press). Our continuing studies of the *T. ulmifolia–E. hegesia* host-plant–herbivore system are examining this variation in the ability of the host plant to liberate hydrogen cyanide, and the interaction of this variation with herbivores and their predators, in order to explore the potential for chemical mediated coevolution and the significance of tritrophic interactions in hostplant-herbivore systems.

In this paper we present the results of experimental studies of the effects of variation in host-plant cyanogenesis on several aspects of the ecology and life history of *E. hegesia*. Specifically, our objectives are to: (1) assess the suitability of the variety of potential host plants that are available throughout the herbivore's range and any oviposition preference between known primary and secondary host plants; (2) determine whether cyanogenesis in *T. ulmifolia* influences female oviposition preference; (3) examine larval food preference to assess whether larvae are capable of detecting and reacting to cyanogenesis in the host plant; (4) determine what effect host-plant cyanogenesis has on the growth and development of larvae; (5) assess the degree to which larvae may sequester cyanogenic compounds from the host plant; and (6) determine if sequestration of these compounds protects larvae and pupae from potential predators.

METHODS AND MATERIALS

All rearing of *E. hegesia*, except that for pupal fate (see below), was conducted in captivity in the greenhouses at York University (Toronto, Ontario, Canada) as detailed in Schappert and Shore (1998). All larvae and butterflies used in these experiments were originally collected as larvae or eggs from five widely spaced localities in Jamaica. We commonly used second- or third-generation laboratory-reared larvae or butterflies for experiments, but on occasion we used individuals from the fourth or later generations.

All experimental plants of *T. ulmifolia* (unless indicated otherwise) were derived from multiple cuttings of 24 plants originating from four seed families of a single population [population A2 of Shore and Obrist (1992)]. The plants used were those derived from the two families with the highest cyanogenesis levels (denoted Cy, mean ± SE: 1050.9 ± 190.4 μg HCN/g dry wt) and those two with the lowest cyanogenesis levels (denoted Acy, mean ± SE: 2.5 ± 4.2 μg HCN/g dry wt). We specifically chose plants from a single population showing wide variation in cyanogenesis so that the plants would be essentially similar in all but their cyanogenesis levels. Multiple cuttings were made of plants from each family, resulting in a large array of acyanogenic and cyanogenic cloned sets. Quantitative tests for plant cyanogenesis were conducted using the modified Lambert method as detailed in Schappert and Shore (1995).

Study Organisms. Euptoieta hegesia is a common butterfly found throughout the Neotropics including many islands of the Caribbean (Brown and Heineman, 1972; Smith et al., 1994). The genus is allied with both the Argynninae and the Neotropical Heliconiinae (Dos Passos and Grey, 1945; Clark, 1947; Ehrlich, 1958; Ackery, 1988; Harvey, 1991; Martin and Pashley, 1992), and recent molecular work suggests that the Argynninae, Heliconiinae, and Acraeinae are monophyletic (Weller et al., 1996; A. V. Z. Brower, personal communication). *Euptoi-*

eta hegesia is most often associated with *Turnera ulmifolia* but also uses a number of cyanogenic *Passiflora* species, particularly *P. suberosa* L. and *P. foetida* L. in Jamaica (Schappert and Shore, 1998) and *P. foetida* in Costa Rica (Smiley, 1983).

Schappert and Shore (1998) provide details of the life history of *E. hegesia*. Briefly, eggs are laid singly on the apical leaves of host plants and hatch in five days. Larvae develop over 12–15 days, and pupal development occurs over eight or nine days, yielding a total generation time of approx. 25–29 days. Larvae are warningly colored and often gregarious (see Schappert and Shore, 1998, and below). They are brick red with black spines until their third instar, when the ground color deepens to maroon and a silver/white dorsal line edged with black and two similar lateral lines appear. Pupae vary from tan to black with silver and gold markings. Adults are orange–tawny butterflies of medium-size with extensive black markings [resembling their close relative, *Agraulis vanillae* L. (Heliconiinae), but with less elongate forewings] and may be considered warningly colored. The undersides of the wings are mottled brown/tan/purple, however, and may be considered cryptic (Chai, 1988).

Turnera ulmifolia L. is a perennial shrub common to marginal habitats throughout the Neotropics (Barrett, 1978; Barrett and Shore, 1987). It generally has discrete, small populations with little potential gene flow among populations due to high selfing rates, limited pollen dispersal by insects, and/or limitations imposed on seed dispersal distances by ants (Barrett, 1978; Baker and Shore, 1995; Belaoussoff and Shore, 1995). It shows a wide range of morphological and reproductive variation (duQuesnay, 1971; Barrett and Shore, 1987; Baker and Shore, 1995; Belaoussoff and Shore, 1995), as well as extensive variation for cyanogenesis in Jamaica (Schappert and Shore, 1995). Variation for cyanogenesis occurs within individual plants of this species as well as across a number of species, taxonomic varieties, and populations in one section of the genus *Turnera* (Shore and Obrist, 1992). The presence of cyclopentenoid cyanogenic glycosides, in addition to morphological, embryological, and recent DNA sequence data, allies the Turneraceae with the Passifloraceae, the Flacourtiaceae, and other members of the order Violales (Vijayaraghavan and Kaur, 1966; Cronquist, 1981; Spencer et al., 1985; Spencer, 1988; Chase and Swenson, 1995).

Potential Host Range. A random sample of freshly hatched larvae was selected from four plants that had each been available to five or more ovipositing females for 4 hr. The larvae were enclosed in groups of five or 10, depending on host-plant size, on mature flowering plants of 17 different species and varieties of *Turnera*, two species of *Piriqueta* (Turneraceae), and five species and one cultivar of *Passiflora* (Passifloraceae) in the greenhouse at York University (see Table 1). All of the Turneraceae (except for *Piriqueta caroliniana*) and Passifloraceae (except for *Passiflora* Pfordtii) occur in different parts of the normal range of *E. hegesia*. To assess the relative suitability of the plants, the larvae

were removed and weighed after 14 days, when the first few larvae had entered their final molt to pupation, and the survivorship of the larvae was recorded.

Oviposition Preference. We assessed female oviposition preference between acyanogenic and cyanogenic plants with a number of experimental approaches (a progressive refinement of our methods, see below) that included short- and long-duration tests with binary or four-plant choice arrays in both small and large enclosures. Small-enclosure tests were conducted in 64-cm × 71-cm × 85-cm-high wooden frame cages with wire screen floors and covered in bridal veiling (Schappert and Shore, 1998). Large enclosure tests were conducted in a modified 3.7-m × 3.7-m × 2-m-high outdoor "dining shelter" in which the opaque polyethylene roof had been replaced with bridal veil to increase light levels. Test duration varied from 1 hr to four days for some four-plant array tests.

All of the tests shared the following characteristics: oviposition plants were selected from a large pool of plants of known cyanogenic status (above) and matched for number of flowers, approximate size, and number of branches and leaves. Females used in tests were observed to have been ovipositing in rearing cages prior to testing. Plants (one Cy and one Acy) were placed in opposite corners of cages for binary choice tests or in each corner but in an alternating pattern, for four-plant arrays (i.e., Cy, Acy, Cy, Acy). Plants within cages were moved at preset intervals (see below) to reduce position effects.

Long-duration, small-enclosure, four-plant array tests of 11 females were conducted within the *Turnera* greenhouse at York University in the fall of 1990. Plants were moved one position counterclockwise daily and tests were terminated once each plant had occupied each corner of the test cage. Eggs were counted and removed from the plants daily and the same plants were reused. Similar, but cumulative (i.e., eggs were not counted and removed daily) and binary choice, tests of eight females were also conducted within the greenhouse in the late summer/early fall of 1990. Plant positions were exchanged daily, and tests were terminated once each plant had occupied each corner of the test cage. These tests may have confused normal host-plant orientation by females because they were conducted within the *Turnera* greenhouse.

Large-enclosure, long-duration, four-plant array tests of seven wild-caught females were conducted at Discovery Bay Marine Laboratory in Jamaica in the summer of 1991. Each female was tested for a single day with eggs counted at the end of the day. Jamaican tests were conducted using cuttings of plants from highly cyanogenic vs. acyanogenic plant populations [high Cy: MANDEV (mean ± SD: 0.18 ± 0.06 OD_{504}), low Cy: FALMTH (mean ± SD: 0.01 ± 0.01 OD_{504})] (see Schappert and Shore, 1995). Cuttings were relocated one position counterclockwise at arbitrary intervals.

A series of oviposition preference tests was conducted in a large enclosure located outside of the greenhouses at York University during July 1993. All tests were 1 hr in duration with four-plant arrays. Thirteen females were

tested in back-to-back replicates with relocation of the host-plant array (all plants moved one position counterclockwise) between replicates. Three females did not oviposit during the tests and were excluded from subsequent analyses. A final series of small-enclosure, short-duration, binary-choice tests was conducted outside of the greenhouses using 18 females in July 1994. Each test lasted 2 hr with up to 11 replicate tests of individual females. Each cage was rotated 180 degrees after the first hour of each test.

Tests were also conducted to assess female preference between cyanogenic *T. ulmifolia* and either *Passiflora foetida* or *P. suberosa*, two commonly used cyanogenic secondary host plants (see Schappert and Shore, 1998) in Jamaica. Binary-choice, short-duration, small-enclosure tests of eight *T. ulmifolia*-reared females (four females for each of the *Passiflora* spp.) were conducted outside of the greenhouse on two consecutive days in the summer of 1994. Six of these females were then prevented from ovipositing (no available plants) until they were provided with either *P. foetida* or *P. suberosa* as their only oviposition substrate in no-choice, small-enclosure, 4-hr tests over the following two days. At the end of the second test, the *Passiflora* spp. in each enclosure was replaced with a single cyanogenic *T. ulmifolia* (to verify that females were still able or motivated to oviposit) and the number of eggs laid in 30 min was recorded.

Larval Food Preference. Larval food choice was assessed with binary-choice leaf-disk tests. Leaves at similar positions on experimental plants 20-mm-diameter disks of cyanogenic and acyanogenic leaves were cut and placed on opposite sides of a 100-mm Petri dish on moistened filter paper. Preliminary tests indicated there was no significant loss of leaf disk mass for up to 10 hr, provided that a high humidity level was maintained within the feeding arena (unpublished data). A larva was introduced between and facing away from the leaf disks. The available tissue in the leaf disks in each dish was matched as closely as possible by disk mass. The larvae used in each test were full siblings in their second and third instars (instars tested separately), half of which had been reared on acyanogenic plants and half on cyanogenic plants. All tests were conducted at York University in an environmental chamber at 28°C under a mixture of warm white and cool white fluorescent lamps. Tests varied in duration because experiment termination was determined by the larvae (i.e., just before any one larva could eat all of any single leaf disk, to maximize disk consumption). Consequently, second-instar tests were of longer duration than those of third-instars.

Leaf disks were photocopied at the end of each test. All disks were scored for the presence or absence of damage and disk areas were digitized from the photocopies. The amount of each disk consumed during the tests was determined by subtracting the unconsumed area from the initial area of 3.26 cm^2. A total of 16 full-sib larval families with 16–20 larvae per sibship, half from each food history rearing group, were tested in long duration tests (>4 hr,

second-instars, five sibships) and short duration tests (<4 hr, third-instars, 11 sibships).

Larval Growth and Development. We assessed the growth and development of *E. hegesia* larvae using six sibships of 20 larvae each. The full sibships were derived from laboratory crosses of butterflies originally sampled as larvae from Jamaica in December 1992. To ensure uniformity of age at the beginning of the experiment, groups of 20 freshly hatched larvae were selected from acyanogenic and cyanogenic host plants that had received eggs within a single 4-hr period. Each larva was reared in a 30-ml white plastic cup with a tight-fitting clear plastic lid in an environmental chamber at York University (28°C and a 13L : 11D cycle). The larvae were fed diced leaves from plants in the treatment group (acyanogenic vs. cyanogenic leaves) to which they had been randomly assigned. The larvae were fed daily in early instars and twice daily in later instars with excess amounts of mixed, diced leaves to reduce potential leaf quality differences that might occur among individual plants or between young and old leaf tissue (Stamp and Bowers, 1990). Cups were cleaned and the increase in mass of each larva was recorded daily (Mettler AE100 electronic microbalance). Larvae pupated readily in the rearing cups and pupal mass was recorded the day following the last molt.

Sequestration of Cyanogenic Glycosides. The possibility that larvae sequester cyanogenic glycosides from their host plants was explored in two experiments. The first examined differences within and between full-sib families reared on acyanogenic vs. cyanogenic plant tissues. Larvae were reared as in the larval growth and development experiments (above) until they were mid-third instar, when they were "purged" on acyanogenic flower petals of *T. subulata* Smith. Purging avoids the necessity of dissecting out the gut contents before testing the larvae for cyanogenesis, although preliminary tests of the gut contents of a small number of dissected larvae revealed no cyanogenic activity from plant matter in the gut. When fed yellow petals, the frass of the larvae changes color to yellow when all previous leaf tissue has been purged from the gut system. Larvae were frozen at −80°C and later tested for cyanogenesis with sodium picrate paper (see Schappert and Shore, 1995, for picrate paper methods).

The second experiment examined the extent of daily sequestration by larvae throughout development. We used a large, mixed-progeny group of larvae resulting from host plants that were available to multiple ovipositing females in each of eight rearing cages for a 4-hr period. A total of 336 freshly hatched larvae were randomly selected and placed, in groups of 12 or 16, on 16 acyanogenic and 16 cyanogenic experimental host plants. One larva per day was removed from each of 10 or 14 of the plants on a rotating schedule. Some larvae were allowed to pupate for rearing purposes. In all, 288 larvae over 12 days of growth were frozen (after purging on acyanogenic flowers) at −80°C for later cyanogenesis testing.

Larvae in this second experiment were tested for cyanogenesis using a modi-

fied Lambert method similar to that detailed for plant tissue in Schappert and Shore (1995). The sole departure from the method was that larvae were not ground in liquid nitrogen but were removed from the freezer and ground immediately. In preliminary experiments, purged fifth-instars were halved longitudinally, each half divided into thirds, and two parts tested in various combinations of "head," "thorax," and abdomen in (1) controls, where there were no exogenous additions of cyanogenic glycoside or β-glycosidase, (2) where there was addition of exogenous glycoside, and (3) where there was addition of exogenous β-glycosidase enzyme. These experiments showed that all larvae possess the enzymes necessary to hydrolyze cyclopentenoid cyanogenic glycosides but revealed considerable variation in the quantity of cyanogenic glycosides present. Cyanogenic glycoside and crude enzyme extractions were made from the experimental host plants, as described in Schappert and Shore (1995). Furthermore, postincubation acidification tests to attempt to liberate additional HCN (Brinker and Seigler, 1989) showed that all available HCN was driven from the larval tissue after 24 hr of incubation at 37°C.

Larval Defense. To assess whether larvae use sequestered cyanogenic compounds in their own defense, experimental presentations of third-instars reared on contrasting experimental host plants were made to an array of 52 *Anolis sagrei* Duméril and Bibron (Squamata: Polychrotidae) lizards obtained from Jamaica, Cuba, and the Bahamas by a herpetological supplier. Anoles were housed individually in 5-liter glass aquaria in environmental chambers at York University. Each aquarium was whitewashed on three sides to prevent behavioral interactions between anoles. The anoles were acclimated for 30 days at 25°C under a 13L : 11D cycle before the experimental presentations began. During the acclimation period, the anoles were fed every two days with crickets dusted in vitamin supplements.

The initial presentation experiments were with anoles that had been deprived of food for 48 hr prior to the tests. Mealworms [larvae of *Tenebrio molitor* (Coleoptera) reared on rolled oats], providing a strong contrast to crickets yet similar in both size and shape to the larvae, were used to control for hunger (see below). Anoles were randomly assigned to two groups: those that would be presented with acyanogenic-reared larvae and those to be presented with cyanogenic-reared larvae. The presentation protocol began with a mealworm being introduced into an anole enclosure approximately 15 cm in front of the lizard. The time taken to attack and consume the mealworm was recorded, as was the behavior of the anole during the attack. If the mealworm was not attacked and consumed within 5 min it was removed. After the mealworm was eaten or removed, a larva was introduced into the enclosure in a similar fashion. The behavior of the anole during the presentation and the time taken to attack and consume the larva was similarly recorded. If the larva was not attacked within 5 min it was removed. Larvae that were attacked and not consumed were removed and their subsequent fate was followed. If the anole failed to attack a mealworm, and subsequently a larva, it was excluded from

the analysis since the lack of an attack could not be differentiated from satiation. Seven days following the initial presentation, a similar but reciprocal presentation (i.e., presentation of a larva reared on host plants of the opposite cyanogenic type) was made to each anole.

Further experiments were conducted with 14 of the anoles to provide an indication of their ability to discriminate between larval host-plant types: a habituation experiment, where each anole was repeatedly presented with a total of four larvae from the same host-plant type over the course of 12 days, and a reciprocation experiment, where each anole was reciprocally presented with a total of six larvae from host plants of alternating cyanogenic status over the course of 18 days. Anoles were allowed to rest for seven days between these experiments and were provided with vitamin-coated crickets on the day following each presentation and every two days in the intervening period. Thus, all presentations were preceded by a 48-hr fast. Finally, after a further three-day interval, each anole was presented with a freshly eclosed adult butterfly.

Fate of Experimentally Reared Pupae in the Wild. Full-sib larvae were reared to pupation under natural temperature and photoperiod regimes in Jamaica in the summer of 1991. They were fed cuttings of wild-collected plants taken from plant populations with contrasting cyanogenesis levels [high Cy: MANDEV (mean ± SD: 0.18 ± 0.06 OD_{504}), low Cy: FALMTH (mean ± SD: 0.01 ± 0.01 OD_{504})] (see Schappert and Shore, 1995). On the day following pupation, pupae were paired (one from a larva reared on cyanogenic, and the other on acyanogenic, leaves), attached by their cremaster to individual small cotton plugs, and placed in a variety of positions in a population of *T. ulmifolia* located 3.2 km west of Discovery Bay Marine Laboratory. A total of 16 pairs of pupae, differing only in the cyanogenesis levels of their host plant, were placed on host plants, on neighbouring non-host plants, and on rocks within 1 m of nearest host plant. The fate of the pupae was recorded daily.

Data Analyses. All statistical analyses except for the analysis of contingency tables were carried out using SAS ver. 6.12 (SAS, 1996). Unbalanced and repeated-measures ANOVAs were carried out using PROC GLM (SAS, 1996) and type III sums of squares. The experimentwise error rate in multiple comparisons was set to $\alpha_{EW} = 0.05$, following the Bonferroni procedure (Kirk, 1982). Contingency tables were analyzed using the program RXC from BIOM-PC (Rohlf, 1984). Cyanogenesis data for the sequestration experiments were log transformed to stabilize variances.

RESULTS

Potential Host Range. Larvae were able to survive for 14 days on a range of species in the Turneraceae and Passifloraceae (Table 1). The larvae generally obtained a greater mass on Turneraceae than on Passifloraceae (mean mass on

TABLE 1. SURVIVAL AND MEAN MASS OF JAMAICAN *E. hegesia* LARVAE REARED ON SPECIES OF *Turnera*, *Piriqueta* (TURNERACEAE), AND *Passiflora* (PASSIFLORACEAE) COLLECTED FROM VARIOUS LOCALITIES

Species	Locality	Cyanogenesis	Survival	Mass [g, mean ± SE] at 14 days
Turnera				
ulmifolia	Jamaica	Acy	10/10	0.320 ± 0.041
ulmifolia	Jamaica	Cy	10/10	0.293 ± 0.025
acuta	Bahamas	Cy	9/10	0.369 ± 0.026
velutina	Mexico	Acy	8/10	0.327 ± 0.028
orientalis	Argentina	Cy	9/10	0.319 ± 0.017
cuneiformis	Paraguay	Acy	10/10	0.243 ± 0.028
subulata	Brazil	Acy	10/10	0.291 ± 0.033
subulata	Trinidad	Acy	9/10	0.304 ± 0.032
scabra	Dominican Republic	Acy	9/10	0.287 ± 0.026
scabra	El Salvador	Acy	10/10	0.195 ± 0.029
scabra	Venezuela	Acy	9/10	0.228 ± 0.022
scabra	Nicaragua	Acy	5/5	0.115 ± 0.013
scabra	Costa Rica	Acy	5/5	0.090 ± 0.013
scabra	Columbia	Acy	0/5	
krapovickasii	Paraguay	Acy	5/5	0.194 ± 0.021
joelii	Brazil	Cy	2/10	0.025 ± 0.022
panamensis	Panama	Cy	5/10	0.039 ± 0.013
Piriqueta				
caroliniana and *cistoides*	Florida/Jamaica	Cy	7/10	0.123 ± 0.027[a]
Passiflora				
foetida	Jamaica	Cy	8/10	0.233 ± 0.034
suberosa	Jamaica	Cy	8/10	0.215 ± 0.030
edulis	Costa Rica	Cy	8/10	0.014 ± 0.002
biflora	Costa Rica	Cy	7/10	0.087 ± 0.033
caerulea	unknown	Cy	3/10	0.006 ± 0.001
pfordtii	unknown	Cy	2/10	0.003 ± 0.001

[a]Larvae fed on plants of both species.

Turneraceae = 0.25 g vs. Passifloraceae = 0.12 g, $t = 5.73$, $P < 0.001$). There was, however, considerable variation in mass and survivorship among the larvae reared on various Turneraceae. For example, no larvae survived on the tetraploid *T. scabra* plants from Colombia, a population sample that has the most densely pubescent leaves. Larvae survived very poorly on *T. joelii* and obtained only very low masses after 14 days. Poor survivorship and low masses were attained on *T. panamensis*, which is the only species of *Turnera* used that is in a different series of the genus (i.e., not a member of the Canaligerae). Larvae survived on *Piriqueta* spp. but achieved low masses (Table 1). For the Passifloraceae (Table 1), the greatest masses and survivorship were obtained using the two species that

TABLE 2. OVIPOSITION PREFERENCE TESTS OF *E. hegesia* IN RELATION TO CYANOGENESIS IN *T. ulmifolia*

Test and enclosure size	Plants (*N*)/ enclosure	Females (*N*)	Eggs/hr (mean ± SE)	
			Acyanogenic	Cyanogenic
Long duration				
Small	4	11	0.61 ± 0.17	1.07 ± 0.40
Small[b]	2	8	1.83 ± 0.46	1.80 ± 0.64
Large[c]	4	7	0.66 ± 0.39	1.22 ± 0.21
Short duration				
Small	4	6	2.83 ± 0.79	3.02 ± 0.65
Large	4	10	2.80 ± 0.71	3.70 ± 0.76
Small	2	18	5.46 ± 0.63	5.43 ± 0.74
Mean		60	2.82 ± 0.35	3.13 ± 0.36

[a]Data were analyzed as a split-plot factorial ANOVA. Test and females nested within test used as the within-block factors and cyanogenesis and its interactions used as between-block factors. There are significant differences among tests ($F_{5,54} = 30.6$, $P < 0.001$), but cyanogenesis is not significant ($F_{1,54} = 1.2$, $P > 0.25$), nor is its interaction with test ($F_{5,54} = 0.3$, $P > 0.92$).
[b]Cumulative eggs laid per female in replicate trials were used in calculating means.
[c]Experiment carried out in large enclosure in Jamaica.

occur on Jamaica, *P. foetida* and *P. suberosa*. Very poor survivorship and very low masses were attained for all the other species. In general, larvae survived and achieved large masses on both cyanogenic and acyanogenic species of *Turnera*. For example, *T. acuta* from the Bahamas is highly cyanogenic, while *T. subulata* from Brazil and Trinidad are entirely acyanogenic (Shore and Obrist, 1992).

Oviposition Preference. We have assessed whether female butterflies discriminate between cyanogenic and acyanogenic plants of *T. ulmifolia* in a number of different experiments, including long- and short-duration tests with binary or four-plant array choices and by using different sized enclosures. There is a slight overall trend towards more eggs being laid on cyanogenic as opposed to acyanogenic host plants although this varies among different tests (Table 2). A split-plot factorial ANOVA, however, reveals significant differences only in numbers of eggs laid among different experimental tests and no significant effect for the cyanogenic status of the host plants (Table 2).

We also carried out oviposition tests comparing *Turnera* vs. *Passiflora* as host plants. The results revealed an overwhelming preference for *T. ulmifolia* (99.3% of eggs laid). No eggs were laid on *P. suberosa* and only two eggs were laid on *P. foetida*, each by a different female. Three of six females subsequently laid an egg on *P. foetida* when presented with the species in no-choice acceptance tests (none were laid on *P. suberosa*). In a total of 8 hr (two 4-hr tests on consecutive days with no oviposition hostplant available between tests) only

TABLE 3. FEEDING PREFERENCE OF SECOND INSTARS OF *E. hegesia* IN RELATION TO HOST-PLANT CYANOGENESIS[a]

Larval family	Larvae per family	% leaf disk consumed (mean ± SE)	
		Cy	Acy
1	15	70.5 ± 7.6	62.6 ± 10.3
2	16	54.2 ± 7.5	45.7 ± 10.1
3	15	63.8 ± 8.7	43.6 ± 9.5
4	15	30.3 ± 6.1	31.3 ± 7.2
5	14	19.4 ± 10.3	39.0 ± 7.7
Mean		48.1 ± 4.2	44.5 ± 4.1

[a]A split-plot factorial ANOVA gave significant differences among family ($F_{4,70} = 7.77$, $P < 0.001$), but cyanogenesis is not significant ($F_{1,70} = 0.30$, $P > 0.58$), nor is family × cyanogenesis ($F_{4,70} = 1.24$, $P > 0.3$).

four eggs were laid on *P. foetida*; only one of these was laid in the second test. In contrast, these six females laid 13 eggs in only 30 min on *T. ulmifolia* following the second test, attesting that the females were highly motivated to oviposit but largely refused the two *Passiflora* species. The results indicate a strong oviposition preference for *T. ulmifolia* as the host plant of choice and, coupled with field observations and relative performance tests (Schappert and Shore, 1998), indicate that Jamaican *Euptoieta hegesia* largely specialize on *T. ulmifolia*.

Larval Food Preference. Larval feeding preference has been assessed in binary-choice leaf-disk experiments for both second- and third-instars. Larvae tested consumed both cyanogenic and acyanogenic leaf tissue. The second-instars showed a preference for cyanogenic plant tissue in three of five larval families; however, there is no evidence for a significant effect of cyanogenesis or a significant cyanogenesis × family interaction (Table 3). Third-instar tests were of shorter duration, and again there is a slight preference for cyanogenic leaf disks but no significant cyanogenesis or cyanogenesis by family interaction effects (Table 4). There is a significant family effect in both experiments, indicating that some larval families consumed more leaf tissue than others (Tables 3 and 4). No association was found between the cyanogenic status of the plants that the larvae were initially reared on and their leaf disk consumption during the experiments.

Larval Growth and Development. The growth of larvae reared on acyanogenic vs. cyanogenic plant tissue revealed no discernible differences (Figure 1). Repeated measures ANOVA of the effect of host plant cyanogenesis on the daily growth of the larvae reveals a significant effect of larval family, significant growth over days, and a family × day interaction that indicates some families grow more rapidly than others. There is, however, no cyanogenesis effect

TABLE 4. FEEDING PREFERENCE OF THIRD INSTARS OF *E. hegesia* IN RELATION TO HOST-PLANT CYANOGENESIS[a]

Larval family	Larvae per family	% leaf disk consumed (mean ± SE)	
		Cy	Acy
6	15	46.8 ± 3.5	43.1 ± 4.2
7	16	49.6 ± 4.3	27.2 ± 6.6
8	14	38.4 ± 11.3	45.0 ± 12.5
9	20	27.2 ± 8.0	40.1 ± 7.7
10	20	43.3 ± 3.9	49.0 ± 4.6
11	20	45.7 ± 4.0	37.7 ± 3.4
12	20	67.5 ± 8.0	54.1 ± 8.4
13	20	56.5 ± 3.9	47.5 ± 5.9
14	20	42.3 ± 3.1	33.5 ± 3.0
15	20	35.7 ± 6.1	22.3 ± 5.8
16	20	44.1 ± 5.5	57.8 ± 4.9
Mean		45.3 ± 1.9	41.7 ± 2.0

[a]A split-plot factorial ANOVA gave significant differences among family ($F_{10,194} = 5.01$, $P < 0.001$), but cyanogenesis is not significant ($F_{1,194} = 1.8$, $P > 0.18$), nor is family × cyanogenesis ($F_{10,194} = 1.7$, $P > 0.08$).

or cyanogenesis × family or higher order interactions involving cyanogenesis (Table 5). The number of days to pupation did not differ between cyanogenic versus acyanogenic fed larvae (mean for cyanogenic fed larvae: 13.8 ± 1.6 days, acyanogenic fed larvae: 13.7 ± 1.3 days; $F_{1,5} = 0.4$, $P = 0.57$), and there was no evidence of family effects ($F_{5,100} = 1.76$, $P = 0.12$) or family × cyanogenesis interaction ($F_{5,100} = 1.21$, $P = 0.31$).

Sequestration of Cyanogenic Glycosides. Full-sib larvae reared on cyanogenic plant tissue had higher cyanogenesis levels than those fed on acyanogenic tissue in all seven larval families tested (Table 6). A two-way ANOVA reveals significant variation between families and significantly greater cyanogenesis in larvae reared on cyanogenic host plants (Table 6). There was no interaction between family and cyanogenesis of host plant. Larvae that were reared on plants with low cyanogenesis levels did, however, liberate some HCN. The mean cyanogenesis level detected in acyanogenic-reared larvae corresponds to 0.013 μmol of HCN, which is approximately the detection limit of the picrate method used here (Shore and Obrist, 1992). The mean for cyanogenic-reared larvae was approximately four times higher at 0.050 μmol HCN.

We used a modified Lambert quantitative cyanogenesis assay (Brinker and Seigler, 1989; see Methods and Materials for modifications) to assess daily sequestration levels of larvae through development. An initial burst of cyanogenesis occurs in young larvae fed cyanogenic host plants (Figure 2). Cyanogenesis

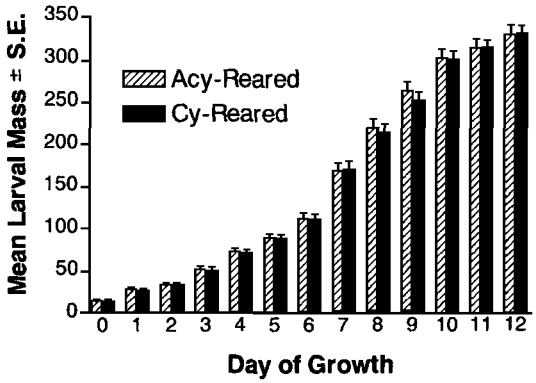

FIG. 1. Growth of larvae reared on cyanogenic (Cy) versus acyanogenic (Acy) leaves of *Turnera ulmifolia*

levels per gram dry weight were subsequently lower than this initial burst but usually greater for cyanogenic- as opposed to acyanogenic-reared larvae (Figure 2). A two-way ANOVA gave significant effects for all terms including cyanogenesis ($F_{1,260} = 24.8$, $P < 0.001$), day ($F_{11,260} = 6.36$, $P < 0.001$) and cyanogenesis × day interaction ($F_{11,260} = 3.37$, $P < 0.001$).

Larval Defense Against Predators. A series of experiments was conducted to determine whether larvae fed cyanogenic as opposed to acyanogenic leaves are afforded protection against predation by *Anolis sagrei*. In the first experiment, 11 anoles did not attack a mealworm or a larvae (so could not be distin-

TABLE 5. REPEATED MEASURES ANOVA OF MASS OF *E. hegesia* LARVAE MEASURED OVER TIME IN RELATION TO HOST-PLANT CYANOGENESIS

Source	*df*	*F*	*P*
Within-subject effects			
Day	7	750.5	<0.001
Day × family	35	7.5	<0.001
Day × cyanogenesis	7	0.1	0.83
Day × family × cyanogenesis	35	1.4	0.21
Error (day)	721		
Between-subject effects			
Family	5	15.4	<0.001
Cyanogenesis	1	0.2	0.63
Family × cyanogenesis	5	1.1	0.39
Residual	103		

TABLE 6. COMPARISON OF HCN LEVELS IN *E. hegesia* LARVAE REARED ON ACYANOGENIC VS. CYANOGENIC HOST PLANTS, BY FAMILY[a]

Larval family	Acy		Cy		t	P
	N	OD_{504}	N	OD_{540}		
1	12	0.039 ± 0.027	12	0.092 ± 0.067	2.49	0.021
2	15	−0.002 ± 0.016	15	0.008 ± 0.026	1.31	0.200
3	10	−0.002 ± 0.016	9	0.047 ± 0.041	3.53	0.003[b]
4	18	0.063 ± 0.045	16	0.139 ± 0.092	3.15	0.004[b]
5	5	0.007 ± 0.012	9	0.024 ± 0.033	1.11	0.290
6	18	0.010 ± 0.012	18	0.042 ± 0.045	2.93	0.006[b]
7	11	0.011 ± 0.014	11	0.044 ± 0.035	2.92	0.008
Mean		0.018 ± 0.024		0.057 ± 0.045		

[a]HCN levels are expressed as means (±SD) optical density measured at 504 nm. Two-way ANOVA gave significant differences among families, $F_{6,164} = 17.3$, $P < 0.001$, a significant cyanogenesis effect, $F_{1,6} = 17.26$, $P < 0.01$, but no family × cyanogenesis interaction, $F_{6,164} = 2.10$, $P > 0.06$. Family was considered a random effect.
[b]Individual comparisons are significant if $P < 0.0073$.

guished from anoles that were satiated), reducing the experimental presentation sample size to 41 presumably naive anoles. The *Anolis* lizards did not discriminate between *E. hegesia* larvae reared on acyanogenic vs. cyanogenic host plants and attacked them at equal rates (Table 7); however, only 56% of anoles that accepted a mealworm also attacked a larva. This suggests that anoles may per-

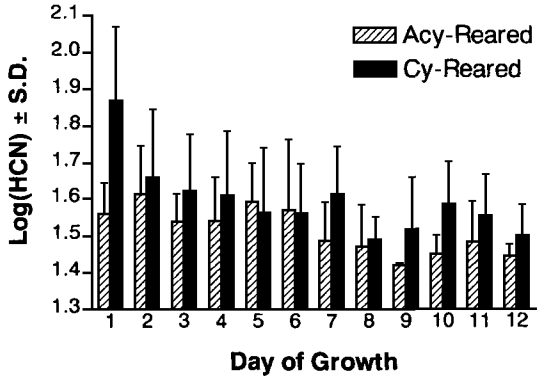

FIG. 2. Mean cyanogenesis levels of larvae reared on cyanogenic (Cy) versus acyanogenic (Acy) leaves of *Turnera ulmifolia*. Samples sizes varied from 9 to 14 larvae per day per treatment.

TABLE 7. RESPONSE OF NAIVE *Anolis sagrei* TO PRESENTATION OF *E. hegesia* LARVAE REARED ON EITHER ACYANOGENIC OR CYANOGENIC *T. ulmifolia*

	Larvae reared on	
	Acy.	Cy.
Attack		
No	9	9
Yes	11	12
Total:	20	21
$G = 0.019$, $P = 0.89$		
56% attacked		
Postattack		
Drop	3	8
Eat	8	4
Total	11	12
$G = 3.48$, $P = 0.055$		

ceive the larvae to be aposematic due to their coloration, their spiny nature, or a combination of both traits (Bowers, 1993). Of the larvae attacked, there is a trend towards a disproportionate number of acyanogenic-reared larvae being eaten as opposed to cyanogenic-reared larvae (Table 7). Importantly, of the 11 larvae attacked but not consumed, seven survived the attack and successfully developed through to pupation. The reciprocal presentation of larvae of the opposite cyanogenic status to the "now-experienced" anoles provided results that were very similar to the results from the naive presentations (Table 8). In this case, the trend towards disproportionate numbers of acyanogenic-reared larvae being eaten as opposed to cyanogenic-reared larvae was significant (Table 8). When the data in Tables 7 and 8 are pooled, 76% of acyanogenic larvae were consumed as opposed to 32% of cyanogenic-reared larvae.

In two additional presentation experiments, where 14 anoles were repeatedly presented with larvae that were reared on cyanogenic or acyanogenic leaves (habituation) or alternately presented with larvae reared on the two types of leaves (reciprocation), anoles appear to be incapable of discriminating between larvae until they were tasted (i.e., attack rates do not differ significantly; habituation: $G = 0.01$, $P = 0.91$, 50.7% attacked; reciprocation: $G = 0.12$, $P = 0.73$, 77% attacked). In both instances a significantly greater proportion of acyanogenic-reared larvae were consumed (habituation: $G = 28.2$, $P < 0.001$; reciprocation: $G = 7.0$, $P = 0.008$). Of 16 larvae that were attacked but not consumed in the reciprocal presentations experiment, 10 survived to pupation. During these experiments, behavioral observations revealed that *E. hegesia* larvae that were dropped by *A. sagrei* often elicited responses of head-shaking, wiping of the jaw against

TABLE 8. RESPONSE OF *Anolis sagrei* TO RECIPROCAL PRESENTATION OF *E. hegesia* LARVAE REARED ON EITHER ACYANOGENIC OR CYANOGENIC *T. ulmifolia*

	Larvae reared on	
	Acy.	Cy.
Attack		
No	10	8
Yes	10	10
Total	20	18
$G = 0.11$, $P = 0.73$		
52.6% attacked		
Postattack		
Drop	2	7
Eat	8	3
Total	10	10
$G = 5.30$, $P = 0.021$		

the floor or with a foreleg, gaping, and/or repeatedly scraping the tongue in and out against the upper jaw.

In a final experiment with the anoles, we presented freshly eclosed adult butterflies to 14 anoles. All 14 of the butterflies were attacked but only six butterflies were eaten after 24 hr (equal numbers of acyanogenic- and cyanogenic-reared adults).

Fate of Experimentally Reared Pupae in the Wild. Paired full-sib pupae that had been reared on acyanogenic vs. cyanogenic plants of *T. ulmifolia* in Jamaica fared poorly. None of the 32 pupae survived more than three days after being placed in the field. Pupae disappeared quickly from the host plants and from positions on nearby rocks but did survive somewhat longer on other vegetation adjacent to or further removed from the host plants. No association between cyanogenesis and survivorship was found. Predators evidently removed pupae since partially consumed pupae were discovered in a few instances, but no acts of direct predation were observed.

DISCUSSION

Our studies of the effects of cyanogenesis in *T. ulmifolia* on host-plant choice by *E. hegesia* have revealed that cyanogenesis: (1) has no effect on host-plant choice by ovipositing female butterflies or by feeding larvae, despite a finding that eggs and larvae of *E. hegesia* tend to occur somewhat more frequently on less cyanogenic plants in Jamaica (Schappert, 1997; Schappert and

Shore, in press); (2) has no effect on larval growth and development; and (3) does not appear to exert a significant effect on the range of potential host plants that could be used by this species throughout its geographic range. Cyanogenesis does, however, have a significant effect on larval survival by protecting them from consumption by *Anolis sagrei*, presumably via the sequestration of cyanogenic glycosides.

Oviposition preference in butterflies is a product of both prealighting (i.e., host choice) and postalighting (i.e., host acceptance) mechanisms (Singer, 1986; Renwick, 1989; Thompson and Pellmyr, 1991). Host choice, that is discrimination between available plants, is exercised through a variety of factors, including plant or leaf size and shape, plant habitat, location or dispersion, and the identification of volatile chemical compounds liberated by the plants (Feeny et al., 1989). Host acceptance (i.e., oviposition), however, depends to a large degree on host plant chemistry, especially the surface chemistry of the chosen host (Renwick, 1989). Ovipositing females do not always make the best host-plant choices (Rausher, 1979), although most studies show a correlation between larval performance and/or survival and oviposition choice (Singer, 1986; Thompson and Pellmyr, 1991; Singer et al., 1994). That *E. hegesia* is capable of discriminating between potential hosts is shown by the restriction of their host choices to members of the Turneraceae and Passifloraceae and by our experiments that reveal marked preference for *Turnera* over *Passiflora*. However, our studies show that cyanogenesis is apparently not a component of oviposition choice.

The growth and development, and ultimately the survival, of larvae is largely dependent on the oviposition choices made by female butterflies (Feeny et al., 1983), but the constitutive nature of cyanogenic glycosides has been suggested to provide no overt cues that could allow herbivores to discriminate among plants (Jones, 1988). Yet, *E. hegesia* does discriminate between available hosts in nature. Larvae are distributed nonrandomly among available *T. ulmifolia* plants within populations, with a highly significant degree of clumping of larvae on single hosts (up to 42 on a single plant) (Schappert and Shore, 1998), suggesting that some plants are preferred over many others that lack larvae entirely. Larval food availability is largely determined by female oviposition choice (Feeny et al., 1983; but see below) and, since *E. hegesia* lays its eggs singly, aggregations of larvae on single plants require that repeated oviposition choices of the same hostplant be made. The cyanogenic status of host plants does appear to influence oviposition choice since plants in nature, which had eggs and larvae, were found to be less cyanogenic, on average (with a significant difference in one population), than plants that lacked larvae (Schappert, 1997; Schappert and Shore, in press). However, these results may be an outcome of the distributional patterns of both butterfly and host plant populations, rather than due to host choice based upon cyanogenesis.

Previous work has shown that variation for cyanogenesis occurs within

and between individuals of *T. ulmifolia* (Shore and Obrist, 1992; Schappert and Shore, 1995) suggesting that it might be beneficial for larvae of *E. hegesia* to move between cyanogenic plants or tissues. Additionally, late-instars of *E. hegesia* are known "wanderers," leaving the host because of early host leaf senescence, defoliation, or other reasons (Faeth et al., 1981; Thompson, 1983; DeVries, 1987; Schappert and Shore, 1998) and must search for and accept new hosts. However, when presented with a choice between cyanogenic and acyanogenic tissue, larvae did not discriminate between the leaf tissues. Larvae do grow and develop reasonably well on *P. foetida* and *P. suberosa* (see also Schappert and Shore, 1998), which further suggests that larval performance may be somewhat more labile than oviposition preference.

Cyanogenesis in *T. ulmifolia* has little or no effect on the relative growth rate of *E. hegesia* larvae. There was an effect of family but no effect of the cyanogenic status of their food and on interaction between treatment and sibship. This is quite different from the findings of studies on other specializing lepidopteran herbivores, which typically perform better on plants containing greater quantities of other defensive compounds (Bowers and Puttick, 1988; Zangerl and Berenbaum, 1993), but has been reported for other lepidopteran and non-lepidopteran herbivores that use cyanogenic host plants (Bernays et al., 1977; Smiley and Wisdom, 1985; Thomas, 1987). For example, Bernays et al. (1977) found that cyanogenesis had a deterrent effect on acridid grasshopper feeding on cassava (*Manihot esculenta*) in the field yet they fed readily, and developed faster and to a larger size, on cut leaves of this plant in the laboratory. If cutting of the leaf tissue was responsible for HCN release that the acridids perceived as a phagostimulant (e.g., Scriber, 1978; Brattsten et al., 1983), then this may be responsible for their finding. However, our use of cut tissue in this study, and any gustatory responses of the larvae to cyanogenesis, did not yield a similar result.

Our previous studies have shown that *E. hegesia* larvae perform better (survival and development time) on *T. ulmifolia* than on either *P. foetida* or *P. suberosa* (Schappert and Shore, 1998), and these data are supported by the oviposition preference of *Turnera* over *Passiflora* reported here. Using larval performance as an index of host suitability, however, also reveals that there are *Turnera* species within the range of *E. hegesia* that are poor larval host plants, as poor as any *Passiflora* species. Trichome density might account for poor survivorship in one instance (*Turnera scabra* from Colombia). *Turnera joelii*, however, has leaves that are virtually lacking in trichomes and yet larval survivorship was extremely poor. This suggests that a number of factors, not just cyanogenesis, are contributing to the poor host quality of some of these species/populations within the Turneraceae.

Larvae of *E. hegesia* do appear to sequester cyanogenic glycosides from cyanogenic *T. ulmifolia* as shown by the prevalence of higher, and in some indi-

vidual sibships significantly higher, HCN levels in larvae that were reared on highly cyanogenic plants. The significant main effect for families in the two-way ANOVA suggests that there is genetic variation in sequestration ability among the larvae, although maternal effects cannot be excluded in this experimental design. Larvae also varied in cyanogenesis during development with younger larvae tending to be more cyanogenic than older larvae, an unexpected finding that suggests larvae actively metabolize the cyanogenic glycosides they receive from the host plant.

The presence of detectable levels of HCN in larvae reared on acyanogenic plants suggests, however, that larvae may be concentrating low levels of available cyanogenic glycosides, which were below our testing limits, from the host plant (unlikely given the apparent active metabolism mentioned above) or that *E. hegesia*, like some of its Acraeine and Heliconiine relatives, may also be capable of synthesizing cyanogenic compounds, possibly linamarin and/or lotaustralin, de novo (Nahrstedt and Davis, 1981, 1983; Davis and Nahrstedt, 1988; Brown and Francini, 1990; Brown et al., 1991). Raubenheimer (1989) has reported that *Acraea horta* (Acraeinae), feeding on the closely related host-plant family Flacourtiaceae, sequesters gynocardin, a cyclopentenoid cyanogenic glycoside, from its host and Helene Engler (personal communication) has recently found that *Heliconius sara* and *H. hewitsoni* also sequester the same compound from their Passifloraceous hosts. Our studies suggest that the larvae of *E. hegesia* may be capable of both de novo synthesis and sequestration from the host plant, a capability that has not yet been reported for any butterfly species (this capability has been previously reported for the moth *Zygaena trifolii*; Nahrstedt, 1988) or for any taxa known to feed on plants that have cyclopentenoid cyanogenic glycosides. Further chemical studies are currently underway to substantiate this finding.

The incorporation of cyanogenic compounds, whether from synthesis or sequestration, did not protect pupae in the field as all were consumed. White (1987) has suggested that pupae are the most vulnerable stage in the life cycle of butterflies since the pupal stage contains not only the highest biomass in the smallest volume but also degraded tissue in the form of "universal biochemical building blocks," which are immediately usable by any potential predator. Pupae of *E. hegesia* are cryptic, varying from tan to black in color and to date we have not found a living pupa in the wild. Hallman (1979), however, reported finding pupae under rocks near host plants. Our attempt to determine if cyanogenesis might protect the pupae in nature under a variety of conditions revealed only that predator pressure at this stage is extreme.

Anolis lizards are known predators of Lepidoptera larvae and adults (Sexton et al., 1972; Boyden, 1976; Johki and Hidaka, 1979; Moermond, 1981; Ehrlich and Ehrlich, 1982; Floyd and Jenssen, 1983; Odendaal et al., 1987; Owen and Smith, 1990; Larsen, 1992; Roughgarden, 1995; Schappert and Shore, 1998).

Our previous studies of the ecology and potential mortality sources of adult *E. hegesia* in Jamaica have suggested that anoles may play a critical role in the regulation of adult butterfly populations (Schappert and Shore, 1998). The studies we report here further suggest that anoles may also be important predators of the larval stage of *E. hegesia* since more than 50% of the anoles tested would attack a larva given the opportunity. That presumably naive anoles also failed to attack almost 50% of larvae when given the opportunity suggests that larvae are at least partially protected by their appearance, although the experimental design used did not exclude satiation from the initial presentation of a mealworm.

The association between the cyanogenic status of the host plant on which a larva had fed and the feeding behavior and response of the anoles provides further evidence that cyanogenic compounds derived from the host plant are responsible for protecting the larvae. Interestingly, if larvae are also synthesizing cyanogenic compounds, it is apparent that the quantity or quality of cyanogenic compounds that they make is insufficient to protect the larvae from anoles. Importantly, more than 60% of larvae that were attacked and not consumed by the anoles survived to reach pupation. A larva that feeds on a cyanogenic host plant is more likely to survive an attack by an anole. While anoles could act as selective agents on butterfly host choice, we have limited evidence supporting the hypothesis that butterflies prefer cyanogenic plants.

Our larval feeding and oviposition preference data suggest that, alone, *E. hegesia* is not an important agent of selection acting on cyanogenesis in *T. ulmifolia*. The possibility exists, however, that coupled with predation preferences for acyanogenic-reared larvae by anoles, there could be selection favoring acyanogenic plants since anoles might preferentially reduce the herbivore load of acyanogenic plants. To determine the selection pressures acting on cyanogenesis would, however, require an evaluation of the effects of the community of herbivores and possibly plant pathogens of *T. ulmifolia* populations.

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COMPARATIVE RESPONSES OF PARASITOIDS TO SYNTHETIC AND PLANT-EXTRACTED NEPETALACTONE COMPONENT OF APHID SEX PHEROMONES

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Abstract—The responses of aphid parasitoids to completely synthetic and plant-extracted nepetalactone, a component of aphid sex pheromones, were investigated. In wind tunnel tests, *Praon volucre* and *Aphidius ervi* responded equally strongly to both plant-extracted and 99% pure synthetic (4a*S*,7*S*,7a*R*)-nepetalactone. In the field, aphid-infested cereal trap plants were used to test the attractiveness of plant-extracted and synthetic (4a*S*,7*S*,7a*R*)-nepetalactone, as well as the synthetic enantiomer (4a*R*,7*R*,7a*S*)-nepetalactone. There was no significant difference in parasitisation levels by *Praon* spp. between plants baited with the synthetic or with the plant-extracted 7*S* isomers. However, the 7*R* isomer was unattractive alone and rendered the synthetic 7*S* isomer unattractive when combined to form a 50% blend of the two. It is concluded that the response of aphid parasitoids to nepetalactone is influenced more by enantiomeric purity rather than the possible presence of plant-related contaminants associated with plant extraction. The results are compared with data on male aphid responses to the compounds, which indicate that plant-derived contaminants can reduce attractancy, and the implications for the manipulation of parasitoids in the field are discussed.

KEY WORDS: Aphid parasitoid, aphid sex pheromone, kairomone, stereochemistry, (4a*S*,7*S*,7a*R*)-nepetalactone, wind tunnel, trap plant.

INTRODUCTION

Oviparous females of holocyclic aphid species release a sex pheromone in the autumn to attract males for mating (Pettersson, 1970, 1971; Marsh, 1972). For the majority of aphid species studied, the sex pheromone has been shown to con-

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sist of either one or a ratio of two cyclopentanoid monoterpenoids (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Dawson et al., 1987; Pickett et al., 1992). In field trapping experiments, female aphid parasitoids of the genus *Praon* (Hymenoptera; Braconidae: Aphidiinae) were attracted to traps baited with nepetalactone (Hardie et al., 1991; Powell et al., 1993), and responses of the generalist parasitoid *Praon volucre* (Haliday) to this component have since been demonstrated in laboratory bioassays and in trap plant experiments in the field (Lilley et al., 1994; Glinwood, 1998; Glinwood et al., 1998). *Aphidius ervi* (Haliday), an aphidiine parasitoid of the pea aphid, was also attracted to nepetalactone in a wind-tunnel bioassay (Poppy et al., 1997). It is probable that aphid parasitoids can use the aphid sex pheromone as a host location kairomone.

In previous studies of both aphid and parasitoid responses to aphid sex pheromone components, these were extracted from the catmint plant, *Nepeta cataria* L. (Laminaceae = Labiatae) (Dawson et al., 1989). However, a range of aphid sex pheromone components and their enantiomers have recently been completely synthesized (Dawson et al., 1996) and tested against male aphids in the field (Hardie et al., 1997). Traps baited with synthetic 99% (4a*S*,7*S*,7a*R*)-nepetalactone captured significantly more male blackberry-cereal aphids, *Sitobion fragariae* (Walker), than did traps baited with the plant-extracted (4a*S*,7*S*,7a*R*)-nepetalactone, while the presence of the enantiomer (4a*R*,7*R*,7a*S*)-nepetalactone significantly reduced catches in the synthetic nepetalactone-baited traps (Hardie et al., 1997). Trapping of males of the bird cherry-oat aphid *Rhopalosiphum padi* (L.) with completely synthetic and plant-derived nepetalactols followed a similar trend (Hardie et al., 1997). However, in this case addition of 98% (7*R*)-nepetalactol to the 99% (7*S*)-nepetalactol to form a 50% blend did not result in significantly reduced catches. This suggests that, for aphids, reduced enantiomeric purity in terms of the 7*S* configuration or contaminating substances of plant origin can reduce the activity of sex pheromone components.

Because attempts are being made to manipulate parasitoid populations using aphid sex pheromone (Powell et al., 1993, 1998), it is important to ascertain whether the plant-extracted nepetalactone is acting as a suboptimal attractant, as appears to be the case for male aphids. Here, the responses of aphid parasitoids to synthetic and plant-extracted nepetalactones are compared in the laboratory and the field.

METHODS AND MATERIALS

Chemicals. Plant-extracted (7*S*)-nepetalactone was obtained from the catmint plant, *N. cataria*, as described by Dawson et al. (1989). Synthetic 99% (7*S*)- and 98% (7*R*)-nepetalactones and a racemic mixture (50% 7*S* and 50% 7*R*) were synthesized from the corresponding citronellol by a previously described route (Dawson et al., 1996).

Wind-Tunnel Bioassay. The flight responses of male and female *P. volucre* and female *A. ervi* were tested in a wind tunnel, as used in previous studies of aphid parasitoid flight behavior (Du et al., 1996, 1997). Briefly, the wind tunnel consisted of a rectangular Plexiglas chamber (90 × 30 × 30 cm) through which an airflow (23 ± 2 cm/sec) was created by an electrically operated extraction fan at one end. Lighting (3600 lux) was provided by incandescent tubes suspended above the tunnel and diffused through a layer of translucent paper.

Parasitoids were taken from laboratory populations reared at 15 ± 1°C with a 16L : 8D photoperiod. *Praon volucre* was reared on the English grain aphid *Sitobion avenae* (F.) feeding on winter barley, and *A. ervi* on the pea aphid *Acyrthosiphon pisum* (Harris) on broad bean (*Vicia faba*). Mummies were removed from plants and placed in small Perspex emergence cages, where emerging adults had access to dilute honey solution and the opportunity to mate. All test insects were 2 days old.

Fifty-eight male and 58 female *P. volucre* and 50 *A. ervi* females were tested in each treatment; it had been determined in previous experiments that male *A. ervi* did not respond to aphid sex pheromone in the wind tunnel. They were released individually into an open-ended, horizontal glass tube (7.5 cm long × 4.5 cm diameter) take-off platform, 10 cm above the floor of the tunnel, within the odor plume. The odor source, 20 cm upwind from the release tube, consisted of a small filter paper target (1 × 2 cm) attached to an upright glass rod. The filter paper was treated with either 10 μl synthetic or plant-extracted nepetalactone (1 mg/ml solution in hexane) or 10 μl hexane (control). For each treatment, the number of parasitoids taking flight and the number that made an uninterrupted straight or casting flight directed toward the filter paper target were recorded. Experiments were performed in a controlled environment room at a temperature of 20 ± 1°C. All treatments were tested at least once per experimental session, and treatment sequences were randomized to account for any temporal changes in parasitoid activity or responsiveness. Means for each session were calculated and subjected to the logit transformation $\ln [P/(1 - P)]$, where P is the proportion of wasps responding, determined by $P = (x + 0.5)/(n + 1)$, x being the number of wasps responding and n the number tested. Transformed means were compared by ANOVA and a least significant difference (LSD) test was applied. Statistics were performed using Statistix (version 1.1; NH Analytical Software).

Field Trap Plant Experiment. The response of *Praon* species parasitoids to plant-extracted and synthetic (7*S*)-nepetalactone, and to synthetic (7*R*)-nepetalactone was tested in the field using aphid-infested trap plants, an approach successfully used in previous experiments (Lilley et al., 1994; Glinwood et al., 1998). The trial took place on the experimental farm at IACR-Rothamsted in southern England between August 20 and September 26, 1997, at the edge of a field from which a cereal crop had recently been harvested. Plastic pots containing 10-day-old winter barley (cv. Puffin) (50 seedlings/pot) were infested with 200 mixed-instar *S.*

avenae virginoparae and left overnight for the aphids to settle before they were exposed in the field for three days. While in the field, pots stood in plastic dishes (23 cm diameter), which contained water to maintain plant quality and to act as a barrier against walking predators, and a second dish was supported by wire above each plant to act as a rain shelter. Plants were checked daily and any aphid predators, excluding parasitoids, were removed. The following treatments were tested: (A) synthetic 98% (7*R*)-nepetalactone, (B) synthetic 99% (7*S*)-nepetalactone, (C) synthetic 50% (7*S*)-nepetalactone: 50% (7*R*)-nepetalactone, (D) plant-extracted (7*S*)-nepetalactone, and (E) control (solvent).

Nepetalactone lures were similar to those used to test aphid responses (Hardie et al., 1997). Nepetalactone (10 mg) was placed in small brown glass vials (08-CPV, Chromacol) in diethyl ether (50 μl) with a small hole drilled into the plastic vial stopper to allow slow release of the pheromone. A single vial was attached to a cane and supported in the soil of the plant pot. Control pots received lures containing only diethyl ether, and all lures were replaced after four weeks.

Trap plants were placed 15 m apart, in a line along the field edge, and two blocks of trap plants were deployed, along different edges of the field. Within a block, each experimental treatment was represented by a single trap plant, and these were arranged according to a quasicomplete Latin square. After each three-day exposure period, the positions of the treatments were rerandomized to account for positional effects. The experiment was repeated a total of 10 times.

After retrieving plants from the field, the number of aphids still present on each plant was recorded. The plants were thoroughly searched and aphid predators, including parasitoids, removed. After two weeks in storage, the number of mummies that had formed on each plant was recorded. The mean number of mummies forming on plants of different treatments was subjected to the transformation $x = \log_{10}(y + 1)$ where x is the transformed value and y is the untransformed value. Transformed means were compared by ANOVA followed by an LSD test using Statistix (version 1.1; NH Analytical Software).

RESULTS

Wind-Tunnel Bioassay. In all treatments, at least 90% of the insects tested took off from the release tube and flew in the wind tunnel. Male *P. volucre* showed no significant flight responses to either plant-extracted or completely synthetic (7*S*)-nepetalactone; only two of a total of 174 males made oriented flights towards the target filter paper. However, both *P. volucre* and *A. ervi* females made significantly more oriented, upwind flights in response to both plant-extracted and synthetic (7*S*)-nepetalactone than to the control treatment (ANOVA; *P. volucre:* sed (standard error of difference) = 0.310, $F = 23.2$, *df* 2,171, $P < 0.05$; *A. ervi:* sed = 0.318, $F = 25.16$, *df* 2,147, $P < 0.01$) (Table 1).

Field Trap Plant Experiment. Analysis showed that the mean number of

TABLE 1. PERCENTAGE OF FEMALE *Praon volucre* AND *Aphidius ervi* MAKING ORIENTED, UPWIND FLIGHTS IN RESPONSE TO SYNTHETIC AND PLANT-EXTRACTED (7a*S*,7*S*,7a*R*)-NEPETALACTONE IN A WIND-TUNNEL BIOASSAY[a]

Odor source[b]	Upwind flight (% ± SE)[c]	
	P. volucre	*A. ervi*
Plant-extracted (7*S*)-nepetalactone	34.5 ± 5.9 a	35.5 ± 4.3 a
99% Synthetic (7*S*)-nepetalactone	31.6 ± 5.3 a	38.5 ± 3.3 a
Control	3.3 ± 2.3 b	5.7 ± 2.6 b

[a]*N* = 58 (*P. volucre*) and 50 (*A. ervi*) for each odor source tested.
[b]Chemicals applied to filter paper target; 10 μl nepetalactone (1 mg/ml in hexane) or 10 μl hexane (control).
[c]Column means followed by different letters are significantly different (ANOVA, LSD test; $P < 0.05$).

aphids recovered from the plants after the exposure period did not vary significantly among the treatments (ANOVA; sed = 2.52, $F = 0.06$, df 4,50, $P > 0.05$). Significantly more mummies of *Praon* species were found on plants baited with completely synthetic 99% (7*S*)-nepetalactone or plant-extracted (7*S*)-nepetalactone than on unbaited control plants (ANOVA; sed = 0.280, $F = 3.56$, df 4,50, $P < 0.05$) (Table 2). The number of mummies that formed on plants baited with either (7*R*)-nepetalactone or the 50% 7*S*/7*R* blend was not significantly higher than the number formed on control plants ($P > 0.05$).

DISCUSSION

Female parasitoids of both *P. volucre* and *A. ervi* were attracted to the nepetalactone component of aphid sex pheromones in the wind tunnel. Although

TABLE 2. MEAN NUMBER OF *Praon* SPECIES MUMMIES FORMING ON *Sitobion avenae*-INFESTED WINTER BARLEY TRAP PLANTS EXPOSED FOR 3 DAYS AT MARGIN OF RECENTLY HARVESTED CEREAL FIELD

Nepetalactone lure	*Praon* mummies (mean ± SE)[a]
99% synthetic (7*S*)-nepetalactone	38.7 ± 9.4 a
98% synthetic (7*R*)-nepetalactone	13.6 ± 3.6 ab
50% 7*S*/50 7*R* blend	12.5 ± 4.8 b
Plant-extracted (7*S*)-nepetalactone	23.6 ± 5.5 a
Control (50 μl diethyl ether)	7.3 ± 2.2 b

[a]Column means followed by different letters are significantly different (ANOVA, LSD test; $P < 0.05$).

female *A. ervi* have previously been shown to respond to plant-extracted aphid sex pheromone components in the wind tunnel (Poppy et al., 1997), this is the first demonstration of in-flight attraction of *P. volucre*, and it confirms previous olfactometer and field trapping results (Hardie et al., 1991; Lilley et al., 1994, Glinwood et al., 1998).

Although the purity of the plant-extracted nepetalactone at C-7 is high (Eisenbraun et al., 1980), there may be unidentified plant-derived substances present that reduced catches of male aphids compared with fully synthetic preparations (Hardie et al., 1997). However, in the wind-tunnel bioassays, both parasitoid species were attracted equally to the synthetic and plant-extracted (7*S*)-nepetalactone, suggesting that any contaminants were not influencing the parasitoid response. The trap plant method also efficiently highlighted differences in the degree of attractiveness between the various compounds tested. The most striking influence on parasitoid attraction was exhibited by the 7*R* isomer, which alone did not significantly increase the attractiveness of trap plants, compared with the unbaited controls, and appeared to nullify the activity of the 7*S* isomer. However, there was no repellency as observed with some other kairomone enantiomers (Dickens and Mori, 1989; Levinson et al., 1990; Phillips et al., 1990). Statistically, the synthetic (7*S*)-nepetalactone was not significantly more attractive than the plant-extracted equivalent, confirming the wind-tunnel results. Thus, the role of possible plant contaminants in the plant-extracted pheromone appears to be less important for parasitoids than for aphids, but parasitoid responses were greatly affected by the enantiomeric purity of the pheromone.

That the presence of (7*R*)-lactone in the racemic mixture inhibited attraction to the (7*S*)-lactone indicates that *Praon* can detect the 7*R* isomer and modify its behavioral response to the 7*S* isomer as a consequence. To elucidate the mechanism involved in this discrimination will require further electrophysiological work on the parasitoid antenna, which responded to plant-derived aphid sex pheromone components in previous electroantennogram studies (Hardie et al., 1993). Behavioral and electrophysiological responses to pheromone enantiomers have received attention in other insect species. The bark beetle predator *Thanasimus dubius* F. (Coleoptera: Cleridae) was found to specifically use one enantiomer of its hosts' aggregation pheromone as a host location kairomone (Payne et al., 1984), and males of the braconid parasitoid *Macrocentrus grandii* (Goidanich) responded preferentially to a particular enantiomer of the female sex pheromone, both in the laboratory and the field (Swedenborg et al., 1994).

The failure of the synthetic aphid sex pheromone to enhance the attraction of parasitoids to the same extent as their aphid hosts means that strategies for aphid parasitoid enhancement, using pheromone lures, which are currently under investigation (Powell et al., 1998), can continue to use plant-extracted pheromone material, which appears to perform optimally and is relatively simple and cost effective to produce. The current study gives further insight into

the dual role of nepetalactone in the chemical ecology of aphids and their parasitoids and suggests that, although this compound is important to both groups, their responses to it have been shaped by different evolutionary pressures.

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SCENT MARKING IN MALE PREMATING BEHAVIOR OF *Bombus confusus*

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Abstract—Premating behavior in the species *Bombus confusus* Schenck, 1859 was studied. The marking habits of bumblebee males were revealed and the use of their labial gland secretion in scent marks was proved chemically. Identical compounds were present both in the labial gland secretion and on male-marked objects. This chemical proof is reported for the first time from natural conditions of a *B. confusus* locality. Males' flight activities, site tenacity, and male–male interactions are described in detail. Contrary to literature reports, male *B. confusus* behavior does not basically differ from that of already known perching bumblebee species.

Key Words—Bumblebees, Hymenoptera, Apidae, *Bombus confusus*, perching behavior, scent marking, site tenacity, male–male interaction, geranylcitronellol, (*Z*)-9-octadecenyl acetate.

INTRODUCTION

The strategies of premating behavior of males in bumblebees fall into three general patterns (Lloyd, 1981): (1) Patrolling behavior, is that in which males establish flight paths with scent marks on prominent objects around the route and fly along these routes awaiting the appearance of females that are attracted by a pheromone. This pheromone is produced by a pair of glands in the head in the cephalic part of the labial gland. (2) Perching behavior is that in which males wait individually at prominent objects (perches), stand on or hover over them, and dart at passing virgin queens (gynes) or other objects that resemble them.

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The perches are prominent objects such as stones, twigs, or dried plants. Males in this category usually use a pheromone, too (Alcock and Alcock, 1983; Williams, 1991), and have extremely enlarged compound eyes. (3) Nest entrance waiting behavior is that in which males, often in groups, wait for emerging gynes right at a nest entrance.

Males of *B. confusus* belong to the group of large-eyed males exhibiting the perching behavior. Bumblebee species with this type of premating strategy occur worldwide; for example, *Bombus mendax* in Europe; *B. asiaticus*, *B. rufofasciatus*, and *B. kashmirensis* in Kashmir Himalaya; and *B. nevadensis auricomus* and *B. griseicollis* in North America. In all these species, scent marking of their perching site was observed (Haas, 1949; Alcock and Alcock, 1983; Williams, 1991). Only *B. confusus* males were claimed to be entirely optically oriented in searching for mates (without use of a male pheromone) (Schremmer, 1972). Thus, this species provided one of the few examples of bumblebee species not using pheromones in premating behavior (Morse, 1982; Free, 1987). However, Hovorka et al. (1998) found recently that this species has a fully developed cephalic part of the labial gland that produces a secretion. The composition of this secretion, with geranylcitronellol and (*Z*)-9-octadecenyl acetate as the main components, has been described (Hovorka et al., 1998), and it was suggested that the males' perches are scent marked.

In perching behavior, as in patrolling behavior, there are two distinct behavioral components—scent marking and flight activity that includes darting flights on moving objects and spontaneous, probably control flights. The marking behavior is exhibited mostly at the beginning of perching behavior each day similar to patrolling behavior. All perching species possess a fundamentally similar mating system. The flight season of the males can last for many weeks and an individual male flies for several weeks. The males select suitable places with one or several perches. The species can differ in site preferences; for example *B. nevadensis* prefers sunny open areas, while *B. griseicollis* selects shaded spots near conspicuous trees (Alcock and Alcock, 1983). Another feature of this behavior is the defense of the perching site. This activity differs from species to species, ranging from the absence of this behavior to heavy fights leading to male injury (Williams, 1991). These differences led to distinguishing new categories in perching behavior: (1) racing and cruising behavior (in cruising behavior a male hovers over his lookout area, while in racing behavior he sits on his perch) without competing for preferred perches, and (2) territorial behavior with competition for perch sites. Schremmer (1972) did not record any male–male interaction in *B. confusus*, did not observe any darting flights to pursue nearby flying insects, and considered all flights spontaneously induced. This would mean that *B. confusus* significantly differs from the other perching species.

A comparison of the chemical composition of the labial gland secretion with the odor of laid marks was previously done in *B. pratorum* and *B. lapidarius*

by Bergman and Bergström (1997) in laboratory conditions. Our study proves the use of the labial gland secretion in scent marks of *B. confusus* males in their natural conditions. This paper also reveals scent marking habits in this species and presents new facts on males' flight activities, site tenacity of males, and male–male interactions. The premating behavior of *B. confusus* is compared with that of other perching bumblebee species.

METHODS AND MATERIALS

Chemical Analyses. We analyzed an extract of a male labial gland, a wash of a marking male's body, a wash of dry flowers (*Cyanus stoebe*) used by a male as a perch, a headspace sample of a marking male's perch (*Cyanus stoebe*), and a wash of male-marked grass straws in the vicinity of a male's perch (within a male's territory).

The labial gland of a *B. confusus* male was dissected and extracted with hexane (50 μl) (Hovorka et al., 1998). Ventral parts of the bodies of three *B. confusus* males were washed with hexane (1.5 ml) and the solution was concentrated to 50 μl prior to analysis. We used two methods for obtaining samples from a male's scent-marked perch: extraction of the perch and a headspace technique for trapping volatiles from a source of odor. Dry flowers used by a male as a perch were extracted in hexane (two flowers in 1 ml of hexane, extraction time 6 hr). The extract was concentrated to 50 μl prior to analysis. The same extraction method was used to obtain samples from males' marked grass straws. For the dynamic headspace, an apparatus described by Jursík et al. (1991) was used. Purified air (flow 14 ml/min, time 18 hr) was blown through a container with the scent-marked flowers (15 flowers) and the surface volatiles were trapped with a charcoal cartridge (3.6 mm ID, 20 mg charcoal; CLSA-Filter, Winterthur, Switzerland). The trapped compounds were eluted from the charcoal with hexane (2 fractions, 2×100 μl) and ether (150 μl). These fractions were combined and concentrated to 30 μl for the final analysis. In all cases, blank samples of unmarked plant material also were prepared and analyzed.

The samples were analyzed on a gas chromatograph with a splitless injector (200°C) and a mass detector (Fisons MD 800), working in electron impact ionization mode. A BPX5 column (30 m $\times$ 0.22 mm, film thickness 0.25 μm; SGE) and helium gas (flow 0.6 ml/min at 50°C) were used for the separations. The temperature program started at 50°C (2 min delay) after which the temperature of the oven was increased to 140°C at a rate of 50°C/min, then to 240°C at a rate of 2°C/min, and finally to 300°C at a rate of 5°C/min. The identification of compounds was based on their mass spectra and retention times. The positions of the double bonds were determined from the mass spectra of dimethyl disulfide adducts as described by Attygalle et al. (1993). The configurations of

the double bonds in 9-octadecenol and methyl 9-octadecenoate were determined (after their chromatographic separation) from the retention times and cochromatography with the corresponding *E* and *Z* standards (180°C isothermally on a DB-1 column (methyl silicone, 30 m × 0.25 mm, film thickness 0.25 μm; hydrogen as carrier gas, flow 1.2 ml/min). Double-bond configurations in unsaturated hydrocarbons were determined from IR spectra measured on Bruker IFS-88 instrument in a KBr micropellet (1.5 mm diameter). The composition of the scent-mark samples was compared with that of the labial gland extract and the body wash.

Behavior. The observations were conducted on the top of a hill (Oblík, elevation 509 m, Central Mountains, Czech Republic), from July 30 to September 17, 1997, in 20 whole-day sessions (July 30; August 4, 6, 7, 10, 12–14, 21–24, 26, 27; September 2–4, 12, 16, and 17; observation period from 8 AM to 5 PM). This study also includes one observation of *B. confusus* males in Pálava, South Moravia (August 14, 1997, elevation 500 m). Males (42 individuals) were individually labeled with plastic tags, usually used for honeybee queens, to enable identification and monitoring of their flight and behavior. The labeling did not influence their behavior in any respect. Within a few minutes after a label has been glued on their thorax, they continued their regular perching behavior. Flight activities were recorded in 15-min periods with randomly chosen males.

RESULTS AND DISCUSSION

Chemical Composition of Scent Marks. Components of the labial gland extract are listed in Table 1 (Hovorka et al., 1998). The composition is compared with the wash of the ventral part of a male's body as well as with the compounds deposited by a male on a perch and grass straws in the vicinity of his perch. Most of the compounds found in the samples were identical (Table 1). The main components of the labial gland secretion [geranylcitronellol and (*Z*)-9-octadecenyl acetate] and the medium-abundant compounds [methyl (*Z*)-9-octadecenoate, tricosane, (*Z*,*Z*)-7,17-pentacosadiene, and pentacosane] were found in all samples analyzed. On the other hand, the two acids (hexanoic and octanoic acid) and their esters with geranylcitronellol, present in the labial gland extract in substantial amounts, were not detected in any other sample nor in the male's body wash. These compounds do not seem to belong to the active pheromonal components as they were not deposited by the males in their territories.

The absence of heavier compounds in the headspace sample of a male's perch may be due to their low volatility. Pentacosane (molecular weight 352) was the biggest molecule detected in this sample. Components present in the labial gland in threshold quantities [(*Z*)-9-octadecenol, geranylcitronellal, octadecyl acetate] were not found in all samples, probably because of their overall low

TABLE 1. COMPOUNDS FOUND IN LABIAL GLAND OF *Bombus confusus* AND THEIR PRESENCE ON MALE-MARKED OBJECTS

Compound[a]	Retention time (min)[b]	Labial gland (%)	Male's body	Dry flower perch		Grass near perch, extract
				headspace	extract	
Hexanoic acid	6.02	0.41	–	–	–	–
Oxtanoic acid	8.23	0.52	–	–	–	–
(Z)-9-Octadecen-1-ol	42.77	0.02	–	+	–	+
Geranylcitronellal	43.56	0.08	–	+	–	+
Methyl (Z)-9-octadecenoate	44.67	4.87	+	+++	+	+
Methyl octadecanoate	45.75	0.18	–	+	–	+
Geranylcitronellol	47.37	29.18	++	++	+	+++
(Z)-9-Octadecenyl acetate	48.96	49.51	+++	+++	++	+++
Octadecyl acetate	49.46	0.04	+	–	–	+
Geranylcitronellyl acetate	51.47	0.42	+	+	–	+
Geranylgeranyl acetate	53.01	0.50	+	+	–	+
Tricosane	53.21	1.41	++	+	++	++
(Z,Z)-7,17-Pentacosadiene	59.23	4.96	+	+	+	+
(Z)-7- and (Z)-9-Pentacosene[c]	59.83	2.23	++	–	+	++
Pentacosane	60.28	0.97	+++	+	+++	+++
Geranylcitronellyl hexanoate	63.38	0.14	–	–	–	–
Heptacosadiene[d]	64.09	0.23	++	–	–	+
(Z)-7-, (Z)-9-, and (Z)-10-Heptacosene[c]	64.63	0.08	++	–	++	+
Heptacosane	64.94	0.02	+++	–	+++	++
Geranylcitronellyl octanoate	67.29	0.27	–	–	–	–

[a]The compounds are listed in the elution order on a BPX5 column (5% phenyl methyl silicone).
[b]For temperature program see Methods and Materials.
[c]Mixture of isomers, not separated.
[d]The double bond positions could not be determined because of the low content of the compound.

concentration compared to the labial gland extract. It is likely that the whole content of the labial gland is not deposited on the marks, because all the samples of marks and the body wash sample were very weak and had to be concentrated before analyses. Chromatographic peaks belonging to the blank sample of flowers and straws were excluded from consideration (Figure 1).

In the male's body wash sample, docosanal was detected in small proportion, but it was not detected in the labial gland extract. It could be an individual difference in the composition of the secretion, as the males used for these two samples were different individuals. However, the composition of the labial gland secretion agreed very well with that described recently for 12 different individuals (Hovorka et al., 1998). The presence of long-chain hydrocarbons in high proportions in the male's body wash sample must be due to the cuticular hydrocarbons washed off together with the labial gland components. Nonacosadiene,

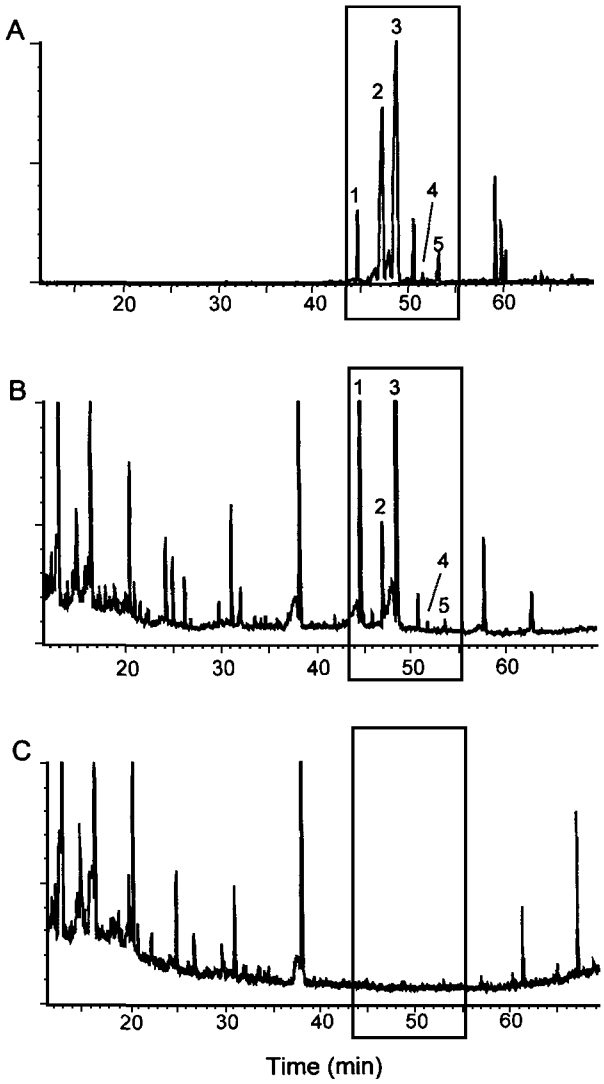

FIG. 1. Chromatograms (recorded on GC-MS): (A) labial gland extract of a *Bombus confusus* male; (B) headspace sample of a male-marked perch (dry *Cyanus stoebe* flowers); (C) headspace sample of nonmarked dry *Cyanus stoebe* flowers (control). The rectangle indicates the area of components of the labial gland secretion [1, methyl (Z)-9-octadecenoate; 2, geranylcitronellol; 3, (Z)-9-octadecenyl acetate; 4, geranylcitronellyl acetate; 5, tricosane] (Hovorka et al., 1998).

nonacosene, and nonacosane, as well as hentriacontadiene, hentriacontene, and hentriacontane were identified in this sample in relatively high amounts, while they were not detected in the labial gland secretion. Also in the washes of marked objects, the content of long-chain hydrocarbons was higher than in the labial gland. The proportion of hydrocarbons in these samples was increased by the presence of these compounds in the blank.

Behavior at Perch Sites. Males chose elevated perches between 10 and 80 cm in height, always in an open sunny area. The favorite perches were dried plants, mostly knapweed (*Cyanus stoebe*), yarrow (*Achillea millefolium*), sometimes bedstraw (*Galium verum*), carthusian pink (*Dianthus carthusianus*), and scabious (*Scabiosa ochroleuca*). The perches were usually not the highest objects within the male lookout area. Individual males differed in number of perches used (Table 2) and in the height of the vegetation at the site. Some males selected perches at sites of relatively high vegetation, where the perches were only minimally prominent, and others at sites covered with tamped grass. Towards the end of the male flight season, probably because of climatic changes (lower temperature), some males perched on the ground ($N = 6$), at least in the first hours of their daily activity. The number of perches used by individual males ranged from 1 to 11 (mean number 4.8, median 3.5, $N = 28$). Nearly in all cases, the male had one primary perch that was highly preferred. Only in relatively few cases were the perches used evenly (Table 2). The perch sites were not distributed evenly at the site. There were places with higher densities of perching males. At particular places, the distances between perch sites of individual males ranged from 2.5 to 11 m, and more often from 7 to 8 m.

All males studied were observed to scent mark their perches and the vegetation in the vicinity. They preferred dead dried plants to green living ones. Beside the already mentioned plant species, they also marked speedwell (*Veronica spicata*), southernwood (*Artemisia campestris*), *Stipa capillata*, etc. The males also occasionally marked stones and at the end of the season, marking the ground (tamped grass) was recorded. The method of scent marking was typical. Males alighted on stalks or grass stems and then walked up and down holding the whole body tight to the substrate. The typical course of scent marking of a grass stem was that a male alighted on the top of a stem and walked downwards. Then he turned around and walked up the same stem or selected another one at its base. The main and side stalks of *Cyanus* were marked in a similar way. In *Cyanus*, males also marked dried florescences, in some cases without marking the stalks. The marked objects were found in a circle of 2–5 m from the primary perch. The number of scent marks laid by one male ranged from 32 to 95 (mean 57.00 $\pm$ 18.19, median 54, $N = 11$; Table 3). Some objects were marked by the same male several times. Scent marking of the territory took 12–26 min (mean 18.82 $\pm$ 4.76 min, median 17 min, $N = 11$). Scent marking was not recorded later in the day. Only one male was seen to mark an object once more about 1 hr after fin-

TABLE 2. PERCHES UTILIZED BY INDIVIDUAL *B. confusus* MALES[a]

Male	Observation date	Perches (*N*)	Percentage utilization of perch[b]		
			No. 1 (main)	Nos. 1+2	Nos. 1+2+3
3	Aug 6	3	81.6	93.8	100.0
11	7	2	89.5	100.0	
11	10	2	65.2	100.0	
12	6	11	27.9	49.5	65.7
13	6	2	74.6	100.0	
15	6	5	73.6	85.1	88.6
18	7	8	53.4	69.3	79.5
19	10	4	73.1	88.4	96.1
19	21	3	89.1	95.9	100.0
24	10	4	60.5	86.4	98.8
27	21	3	66.7	92.9	100.0
27	22	3	52.4	92.4	100.0
28	22	7	63.4	88.9	94.8
28	26	6	53.1	79.6	93.7
30	24	10	36.9	63.8	81.4
30	26	6	39.8	72.8	94.4
31	26	1	100.0		
32	26	3	75.8	91.0	100.0
33	26	5	54.1	87.9	93.3
34	24	4	49.0	89.4	98.1
34	26	1	100.0		
35	Sep 2	1	100.0		
35	12	1	100.0		
36	2	2	98.0	100.0	
37	16	6	38.5	60.0	69.5
38	16	4	78.6	95.3	97.7
39	2	5	59.2	74.5	87.8
39	12	1	100.0		

[a]Data based on three 15-min observation periods.
[b]Percentage utilization calculated from the frequency of landing on the respective perches.

ishing his marking behavior. Possibly this may have been a behavioral response to the appearance of a conspecific male in his territory. Thus, *B. confusus* is a species that marks perch sites at the beginning of the daily activity only, similar to *B. nevadensis auricomus* (Alcock and Alcock, 1983). This may be the reason why Schremmer (1972) did not observe this behavior. The labeled males were seen repeatedly at the same perch sites for a period ranging from 2 to 26 days. In several cases, we labeled the male-marked objects. These objects were either used repeatedly by the same male, or the male marked other objects close to those used previously. The pattern of site marking behavior remained the same throughout the whole season.

TABLE 3. MARKING BEHAVIOR OF INDIVIDUAL *B. confusus* MALES

Male	Observation date	Perches (*N*)	Objects marked (*N*)	Marks laid (*N*)	Marking time (min)
28	Aug 22	7	53	69	16
28	26	6	58	71	25
30	26	6	26	52	16
30	Sep 2	6	37	53	23
32	Aug 27	3	56	67	18
33	27	5	29	36	15
34	Sep 3	1	10	33	14
36	2	2	45	54	17
39	4	1	48	95	26
42	16	1	35	65	25
41	17	1	19	32	12
Mean value			37.82 ± 15.01	57.00 ± 18.19	18.82 ± 4.76

Males appeared at their perch sites between 08 : 45 and 10 : 35 hr. On cooler days without sunshine, initial appearance was delayed (11 : 00–14 : 00 hr). The daily flight activity ended between 17 : 00 and 18 : 00 hr. Males were not at their sites for the whole time, and they sometimes disappeared for several minutes (6–20 min, data based on the observation of three males), probably to feed. Males spent nights mostly outside their territories. Only three males (each only once) were observed to spend the night at their sites.

After appearance in the morning, males combed their hairy coat, especially on the underside, alternately with all pairs of legs. The combing was followed by regular pumping of the proboscis. This lasted for 10–15 min. Thereafter they began to mark their perch sites immediately. After marking, males began flights from their perches. These flights were pursuits, spontaneous control flights, or in some cases, interactions between conspecific males. During the last phases of marking, males often began to respond to flying objects and pursued them. Pursued objects were other species of bumblebees, cuckoo bumblebees, flies, butterflies, and even house martins (*Delichon urbica*). Pursuing of birds was also recorded for two North American bumblebees (Alcock and Alcock, 1983). The numbers of flights per minute ranges from 2.8 to 10.2 (mean 6.28 ± 1.82), based on 41 15-min samples, between 10 : 50 to 14 : 30 hr. The flight activity seems to depend on temperature and wind conditions. The flights induced by flying insects represented 34.2–70.8% of total flight activity (mean 51.52 ± 9.95), based on 24 15-min samples. The rest represented spontaneously induced control flights and shifts between perches. Thus, males of *B. confusus* seem to be more active than their North American counterparts with a mean number of flights of 2.2–2.4 flights per minute (Alcock and Alcock, 1983).

Male–Male Interactions. When a male did chase a false target, he returned

immediately to his perch. A different behavior was observed when he met a conspecific male. Such contact resulted at least in an extended chasing flight. Beside accidental encounters, we have recorded regular interactions, especially between males perching close to each other. The critical distance seemed to be 3–4 m, depending on the character of habitat.

Competing for territories was another reason for interactions. Males appeared continually during the season. The entire history of interactions within the area remains partially unknown due to the interrupted observations. We have observed attempts to invade an already occupied perch site or to select new sites close by. Interactions were very intense when two males tried to occupy the same perch site at the same time. Males usually engaged in a series of chasing flights (up to 10 in a row), alternately pursuing each other. Chasing flights alternated with circling flights over a male's own perch site. Males attacked all passing males and also responded to males sitting on nearby perches. We have seen males knock their opponents to the ground (both from the air and from a perch). The males observed by us did not have damaged limbs or wings, in contrast to the Asian territorial species (Williams, 1991).

The intensity of male–male interactions varied. Most often males interacted periodically, broken by periods when both males pursued flying objects undisturbed by each other. Only in one case were the interactions so intense that males did not pay attention to other flying objects. We observed eight pairs of interacting males during the season—two invasions and six interactions between closely neighboring males. We recorded two changes in perch site occupation (both invasions) and three cases of moving to a new perch site that placed the males further away (6–9 m) from their opponents.

Close neighbors or competitors used a different number of the same objects for marking. A relatively high number of marked objects in their overlapping territories made it difficult to determine the proportion of shared marks. In one case we observed a male perching in the territory of another male without marking. In another case, two interacting males had, among their marked objects, one in common. In a third pair of neighboring males that shared one of the perches, one male marked the area around his three perches including those of his counterpart (67 marks). The second male appeared later and marked a smaller area in close to his five perches, and he marked objects previously marked by the first male (36 marks). The first male did not display any behavioral response. No males were seen to mark the same object simultaneously. It is not clear whether the males are able to recognize their individual odor and if marking an object previously marked by another male was overmarking or only accidental use of the same object. The chemical composition of individual males does not show great differences (Hovorka et al., 1998).

Preliminary experiments helped to resolve this problem. We tried to change the characteristics of the most frequently used perches of several males (N =

6) by adding a 1-cm-diameter circle of filter paper impregnated with 10 μl of solution containing 5 μg of the main secretion component, (*Z*)-9-octadecenyl acetate. These optical and chemical changes of the main perch caused no perceptible behavioral response of the male. According to the literature, the labial gland secretion is used by males mainly for attracting virgin queens to their territories (Bergman, 1997 and references therein). Queens, however, were rarely seen in the area. In spite of the literature report of males responding to their marks (Awram, 1970), we have not obtained any response that could be clearly interpreted this way. To resolve this problem, more detailed observations are needed.

Male–Female Interactions. Only three attempts to copulate were recorded, and all of them were unsuccessful. The first attempt was recorded on August 6 at 14 : 09 hr. A male knocked to the ground a female feeding in his territory and tried to mate. The second two attempts were observed on September 12, between 14 : 00 and 15 : 00 hr. One male was observed to pursue a female from a long distance and to knock her to the ground. The second couple was found on the ground loudly buzzing. Each of the females got away from the male and flew away.

CONCLUSION

The presence of the components of the labial gland secretion on male-marked objects shows that the labial gland secretion of *B. confusus* males is used by males to mark their territories. The observations of the premating behavior of *B. confusus* males show a daily behavioral pattern similar to other perching species. Based on the observed male–male interactions, *B. confusus* seems to be an intermediate case in Williams' classification, probably exhibiting a less pronounced form of Williams' territorial behavior (Williams, 1991). Our results on *B. confusus* seem to support the idea that premating behavior of perching bumblebee species belongs to the category of "lek behavior" in polygyny mating systems (Thornhill and Alcock, 1983).

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