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The Effect of Molecular Structure on Olfactory Discrimination by the Parasitoid *Microplitis croceipes*

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Abstract

Flight chamber experiments were conducted to examine the capacity of the larval parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) to learn to distinguish between structurally related aliphatic alcohols differing in the carbon chain-length and the position of the functional group, and between an alcohol and the respective aldehyde. The parasitoid's ability to discriminate between the components depended on the chain-length of the alcohol to which they had been conditioned. Discrimination improved with increasing difference in carbon chain-length, e.g. the parasitoids made clear distinction between 1-hexanol and 1-octanol. *Microplitis croceipes* could also distinguish different isomers of six-carbon alcohols on the basis of the position of the alcoholic group as well as between 1-hexanol and 1-hexanal. The learning abilities of *M. croceipes* correspond to the specificity of antennal odour receptors towards aliphatic alcohols and aldehydes in previous electrophysiological studies of *M. croceipes* and other insects. Differences in perception or processing of single compounds might reflect differences of their ecological relevance.

Introduction

Parasitoids use odours as they forage for sugar sources, or to locate insect hosts for the purpose of oviposition. Searching behaviour often involves upwind flight toward odours emitted by the resource. In the case of food foraging, these can be flower volatiles (Wäckers, 1994), while during host-search parasitoids may employ volatiles from the host itself, its faeces, host-associated microorganisms, or the plants on which the host lives (Vinson, 1991). A limited number of odours evoke innate flight responses in inexperienced parasitoids (Wäckers, 1994). Parasitoids can usually extend their array of foraging cues by learning (Vet and Dicke, 1992). It has been shown that parasitoids can learn to respond to new compounds by associating them with either a food reward (Lewis and Takasu, 1990) or the host (Papaj and Lewis, 1993). Associative learning of host-associated information may occur during adult emergence, when the egressing parasitoid is exposed to the remnants of its host (Herard *et al.*, 1988; Caubet and Jaisson, 1991; Cortesero and Monge, 1994). Alternatively, parasitoids may associate chemical, as well as visual information experienced during host encounters (Lewis and Tumlinson, 1988; Turlings *et al.*, 1989; Wäckers and Lewis, 1994; Vet *et al.*, 1995).

Despite the broad range of studies on parasitoid learning, little is known about the specificity of the learned response, i.e. the ability of parasitoids to distinguish between conditioned and unconditioned stimuli of similar molecular structure (Vet *et al.*, 1998). Plants emit numerous volatiles, featuring various functional groups and ranging in structure from short, straight carbon chains to complex multi-ring sesquiterpenes (Visser, 1979; Knudsen, *et al.*, 1993). Relevant odour sources might be characterized by subtle differences in volatile chemistry, e.g. volatile plant alcohols and aldehydes varying only in their chain-length or active group. Thus, a high level of odour learning specificity can be critical to the success of parasitoid search.

We chose *Microplitis croceipes* for our experiments as this species has served as a model of parasitoid learning paradigms. *Microplitis croceipes* is a parasitoid of *Helicoverpa* and *Heliothis* spp., whose larvae feed on >100 plant species from different plant families (Fitt, 1989).

Six-carbon alcohols and aldehydes play an important role as 'green leaf volatiles' in the orientation of phytophagous insects to their host plants and of predatory insects and parasitoids to their predatory hosts (Loughrin *et al.*, 1994; Turlings *et al.*, 1998). To examine whether females of *M. croceipes* are able to discriminate between similar alcohols and aldehydes, we trained females to a single compound and subsequently challenged them to discriminate between the conditioned compound and an alternative. The alternatives were: (i) alcohols with a different chain-length; (ii) alcohols with the same chain-length, but with the active chemical group at a different position; and (iii) alcohols and aldehydes with the same chain-length, but different active group. The parasitoid's reaction was tested with dual-choice tests in flight chamber bioassays.

Materials and methods

Insects

Heliothis zea was reared as described previously (Burton, 1969) at 28°C, 50–70% relative humidity (RH) and a 16L:8D photoperiod. *Microplitis croceipes* was reared on *H. zea* larvae according to a previously published method (Lewis and Burton, 1970). The parasitoid females were held with males, water and a honey supply in 30 × 30 × 20 cm acrylic cages. Three-day-old mated females without oviposition experience were used in the experiments.

Flight chamber

The flight chamber used in the experiments was similar to that described in an earlier study (Drost *et al.*, 1988). All flight responses were tested at 25°C, 50% RH, a wind speed of 70 cm/s and a light intensity of 2000 lux.

Conditioning

Females of *M. croceipes* were allowed to contact fresh frass (~20 mg) of artificial-diet-fed *H. zea* larvae provided on a filter paper (9 cm in diameter) for a period of 30 s. Antennal contact with host frass serves as the unconditioned stimulus for *M. croceipes* in associative learning of volatiles (Lewis and Tumlinson, 1988). To condition female parasitoids to a novel odour, 0.5 µl of the compound was applied to a 0.5 × 0.5 cm filter paper which was subsequently placed in a glass pipette. While parasitoids were contacting the frass, they were concurrently exposed to the volatiles emitted from the odour source. For this purpose the odour was blown over their antennae through the odour-laden pipette at a rate of 40 ml/min.

Odour compounds

All tested compounds were purchased from Sigma (St Louis, MO). One group of parasitoids was trained to 1-hexanol, while another group was trained to an odour alternative. This alternative was one of a range of saturated primary alcohols (C4–C10), for chain-length discrimination, 2-hexanol or 3-hexanol (differing with regard to the position of the functional group), or 1-hexanal (differing with regard to the type of functional group). As the last three compounds have a similar vapour pressure compared to 1-hexanol, it can be assumed that parasitoids were exposed to comparable numbers of molecules during conditioning and testing.

Testing

Fifteen minutes after conditioning, parasitoids were introduced into the flight chamber using a 2 dram shell at a position 80 cm downwind of the conditioned odour and an unconditioned alternative. The two odours were presented on strips of filter paper (1 × 2 cm) on each of which 0.5 µl of the respective compound had been placed. Both filter papers were attached to a glass pipette placed vertically on a stand and spaced 12 cm apart. Each insect was given three attempts to complete an oriented flight by landing on one of the odour sources. Uncompleted flights were recorded as well. Females that did not make a choice after three trials or that did not take off after 5 min were recorded as 'no-choice'. All experiments were conducted with 30 different females, 15 of which had been conditioned to each of the odour alternatives. Exceptional to this were experiments with 1-hexanol and 1-hexanal, which were conducted with a total of 40 parasitoids. In order to avoid possible diurnal variation in response, data were collected daily from several insects in each of the different combinations. Differences in choice were tested by χ^2 statistics.

Results

Discrimination relative to differences in chain-length

The parasitoids did not learn to discriminate between 1-hexanol and its direct neighbours 1-pentanol and 1-heptanol (Figure 1). However, at a difference in chain-length of two carbon atoms, the parasitoids were more successful, as they showed a tendency to distinguish between 1-butanol and 1-hexanol, and made a clear distinction between the trained and the untrained alcohol 1-octanol versus 1-hexanol. Success of discrimination dropped strongly when 1-hexanol was pitted against 1-nonanol or 1-decanol. None of the parasitoids trained to 1-hexanol landed at the filter papers treated with 1-nonanol or 1-decanol. Training the parasitoids to 1-nonanol or 1-decanol yielded very few complete flights in the test.

Discrimination based on position of functional group

Following experience with 1-hexanol, 2-hexanol, or 3-hexanol, parasitoids also proved to distinguish effectively between volatiles on the basis of the position of their functional group. Parasitoids trained to 1-hexanol made 50 and 70% of their landings on this odour source when pitted against 2-hexanol and 3-hexanol, respectively. Parasitoids trained to 2-hexanol or 3-hexanol, on the other hand, exclusively flew to the trained component and ignored 1-hexanol (Figure 2).

Discrimination based on type of functional group

Parasitoids were successful in learning to discriminate between 1-hexanol and 1-hexanal (Figure 3). All but one of the landings by parasitoids that had been trained to

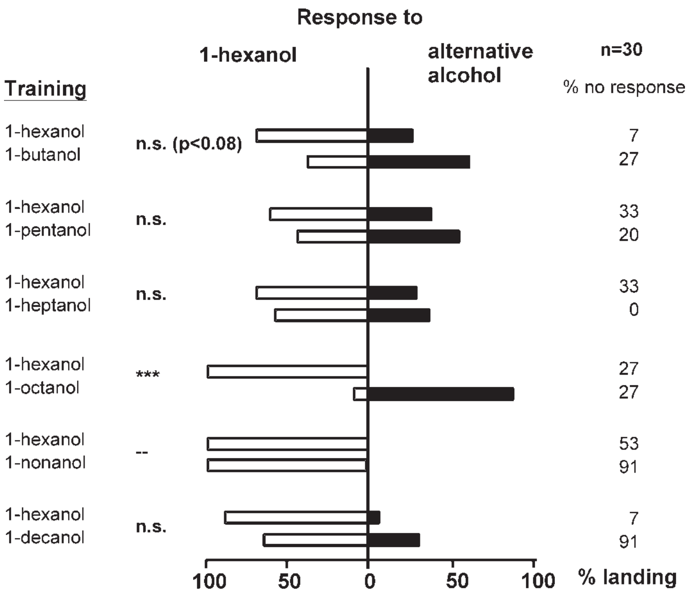

Figure 1 Responses of *M. croceipes* females trained to 1-hexanol (n = 15) or an alternative primary alcohol with different chain-length (n = 15) in flight chamber choice experiments. Bars indicate the percentage of completed flights to 1-hexanol (white bars) or the alternative primary alcohol (black bars). Asterisks indicate a significant difference in the choices between each two differently trained groups (χ^2 test, ***$P < 0.001$).

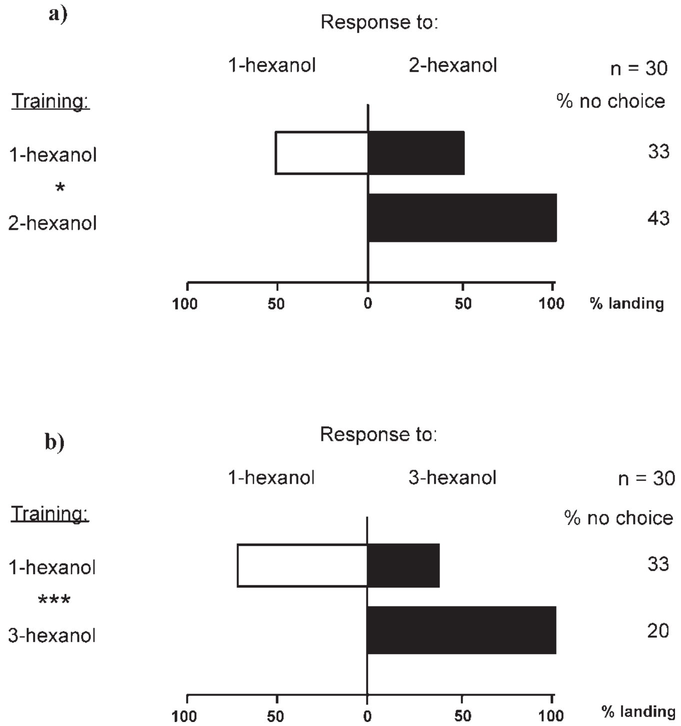

Figure 2 Responses of *M. croceipes* females trained to **(a)** 1-hexanol (n = 15) or 2-hexanol (n = 15) and **(b)** 1-hexanol (n = 15) or 3-hexanol (n = 15) in flight chamber choice experiments. Bars indicate the percentage of completed flights to 1-hexanol (white bars) or the alternative alcohol (black bars). Asterisks indicate a significant difference in the choices between the two differently trained groups (χ^2 test, *$P < 0.05$; ***$P < 0.001$).

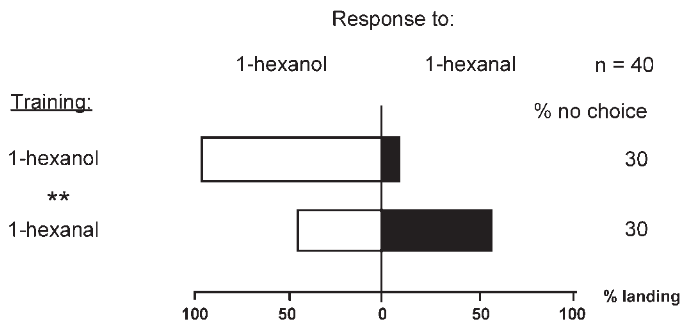

Figure 3 Responses of *M. croceipes* females trained to 1-hexanol (n = 20) or 1-hexanal (n = 20) in flight chamber choice experiments. Bars indicate the percentage of completed flights to 1-hexanol (white bar) or 1-hexanal (black bar). Asterisks indicate a significant difference in the choices between the two differently trained groups (χ^2 test, **$P < 0.01$).

1-hexanol were on the 1-hexanol-treated filter paper target. This clear-cut choice differs significantly from the equal distribution by parasitoids trained to 1-hexanal (Figure 3).

Discussion

In this study, the learning and discrimination abilities of parasitoids were investigated by comparing their responses to 1-hexanol and structurally similar compounds. In conditioned honey bees and sphinx moths, the magnitude of the response to an unconditioned odour depends on the structural similarity between the conditioned and the novel odour compound (Smith and Menzel, 1989a,b; Getz and Smith, 1990; Daly *et al*., 2001).

Discrimination based on differences in chain-length

The results of this study show that parasitoids possess a well-developed ability to discriminate between aliphatic alcohols. Furthermore, we have shown that the ability of *M. croceipes* to learn to differentiate between alcohols depends on difference in chain-length. A difference of at least two C-units appears to be required for successful discrimination in our experiments. Such a negative correlation between discrimination performance and structural similarity in terms of differences of carbon chain-length was also found in humans for aliphatic alcohols, aldehydes, carboxylic acids, aliphatic ketones and acetic esters (Laska and Teubner, 1998, 1999; Laska and Hübener, 2001). It has been shown (Li *et al*., 1992) that electrophysiological responses of antennal odour receptors in *M. croceipes* are maximal for the green leaf volatile 1-hexanol and decline as the carbon chain-length increases or decreases. Also, several other insects show a relationship between the electroantennogram (EAG) amplitude and carbon-chain-length. Other researchers (Schofield *et al*., 1995), working with primary aliphatic alcohols, found that EAGs of the stable fly *Stomoxys calcitrans* increased with carbon chain-length to 1-octanol. Adult boll weevils (*Anthonomus grandis*) showed a maximal response to the C6 and C7 molecules of aliphatic alcohols and aliphatic aldehydes (Dickens, 1984). In the Mediterranean fruit fly (*Ceratitis capitata*), EAG responses were highest in response to stimulation by C7 aliphatic alcohols, aliphatic aldehydes and aliphatic acids, and C5 in the case of aliphatic acetates (Light *et al*., 1988). These EAG hierarchies lead to three interpretations: (i) a group of receptors has a higher affinity for a molecule with a certain chain-length; (ii) there are more receptors for a specific molecule; (iii) EAGs for a specific chemical summate in such a way that the recorded potential is greater for that chemical. Although EAGs indicate which chemicals an insect can smell, no conclusions about the insect's behaviour towards those chemicals can be drawn from these recordings. Behavioural studies are necessary to identify the biological function of a compound and learning bioassays can give information as to whether behavioural responses to certain compounds can change with experience.

None of the *M. croceipes* females that were trained to 1-hexanol landed at the filter papers treated with 1-nonanol or 1-decanol. Also, hardly any parasitoids trained to 1-nonanol and 1-decanol completed any flights, indicating that the parasitoids cannot learn to respond to 1-nonanol and 1-decanol. However, the antennae of *M. croceipes* and *M. demolitor* respond to 1-nonanol and 1-decanol to a comparable degree as to 1-butanol or 1-pentanol (Ramachandran and Norris, 1991; Li *et al*., 1992). Since data from single cell recordings are lacking in *M. croceipes*, it cannot, as yet, be determined whether our results indicate differences in higher-order processing of incoming peripheral information or in the perception of these primary C-9 and C-10 alcohols.

Discrimination based on position of functional group

Asymmetric responses were obtained from *M. croceipes* trained to 1-hexanol, 2-hexanol, or 3-hexanol. While parasitoids trained to 1-hexanol also landed on targets treated with the other alcohol, they clearly distinguished between the alcohols when trained to 2-hexanol or 3-hexanol. (For a discussion of asymmetric responses see below.)

Gustavsson *et al.* (Gustavsson *et al*., 1995) showed that analogues of a turnip moth sex pheromone elicited different electrophysiological single-cell activities based on the position of an oxygen atom replacing a methylene group. They concluded from molecular mechanics that the bioactive conformation is responsible for this effect. The position of the functional groups of different plant volatiles had significant effects on the magnitudes of the EAG responses of the cherry fruit fly *Rhagoletis cerasi* (Raptopoulos *et al.*, 1995).

Discrimination of the functional group

Microplitis croceipes showed asymmetric flight responses in the discrimination of learned aldehydes and alcohols. While 1-hexanol trained parasitoids clearly preferred this alcohol to the aldehyde, parasitoids trained to 1-hexanal showed no difference in their response. This indicates that the parasitoids have an innate preference for the alcohol. Even though conditioning with 1-hexanal did not reverse this preference, it did result in a significant shift in the parasitoid's response towards the aldehyde.

Vet *et al.* (Vet *et al.*, 1998) showed that the parasitoid *Leptolinia heterotoma* can differentiate between C6 compounds with different functional groups. The parasitoids preferred *cis*-3-hexen-1-ol to 1-hexanol and 1-hexanal when they were trained to *cis*-3-hexen-1-ol. But they generalized to 1-hexanal when the learned *cis*-3-hexen-1-ol was not present.

In honey bees, conditioning experiments with alcohols and aldehydes showed that alcohols elicited stronger learned responses than their corresponding aldehydes (Smith and Menzel, 1989b; Smith, 1991). Honey bees conditioned to mixtures of an aliphatic aldehyde and an alcohol showed asymmetric response patterns in proboscis extension (Smith and Cobey, 1994). The response to the aldehyde was much stronger than to the alcohol. The response to the alcohol was much lower when the bee was trained with the alcohol and the aldehyde than when the bee was trained to the same alcohol in the background of another odorant. Honey bee studies of blocking show that behavioural acquisition in response to one component can be hindered or blocked by pre-training with the other component (Smith and Cobey, 1994).

Greater antennal responsiveness to leaf alcohols than to their aldehyde analogues has been found for the Colorado potato beetle and the boll weevil (Visser, 1979; Dickens, 1984), suggesting that differences in the receptor population might explain the higher salience of 1-hexanol. However, others (Hartlieb *et al.*, 1999) showed that some odours were more salient than others in the moth *Spodoptera littoralis*, despite the fact that fewer receptors were present for the salient odour. The authors concluded that the differences in salience might be due to differences in the central nervous system or central processing.

It is not known why some insects perceive certain compounds better than others, but there is evidence that differences in the receptor populations cause this phenomenon. Carbon chain-length, degree of saturation and type and position of functional groups all have a significant effect on the magnitude of EAG response in insects from different orders (Dickens, 1984; Light *et al.*, 1988; Cork, 1994; Raptopoulos *et al.*, 1995; Schofield *et al.*, 1995). On the olfactory receptor level, other researchers (Liljefors *et al.*, 1984, 1985, 1987; Bengtson *et al.*, 1990) have shown a close relationship between the molecular structure and the response of the olfactory receptor for sex pheromone components. Single sensillum studies on plant-odour-detecting neurons suggest that most of these neurons are narrowly rather than broadly tuned and respond only to one or two closely related compounds (Barata *et al.*, 2000, 2002;

Rostelien *et al.*, 2000), thus providing a basis for sensitive odour discrimination (Todd and Baker, 1999). However, although there is a potential for discrimination at the receptor level, the role of central information processing is also important. It has been shown (Stopfer *et al.*, 1997) that discrimination of similar compounds (1-hexanol and 1-octanol) depends on central events (neural synchronization).

The findings of the present study provide evidence of a well-developed discrimination ability for aliphatic compounds in the parasitoid *M. croceipes*. Carbon chain-length and type and position of functional groups all have significant effects on parasitoid learning and discrimination of odours in the context of host finding. The differences in perception and/or processing might reflect the different ecological relevance of the single compounds for *M. croceipes*. Six-carbon alcohols and aldehydes play an important role as 'green leaf volatiles' in the orientation of phytophagous insects to their host plants and of predatory insects and parasitoids to their prey (Loughrin *et al.*, 1994; Turlings *et al.*, 1998). Parasitoids and predators of herbivores might have learning predispositions for certain plant components that are emitted during herbivore feeding (Vet *et al.*, 1990; Steidle and van Loon, 2002). Interference of trained compounds with those volatiles might lead to the asymmetric learning effects described above.

Other work (Takasu and Lewis, 1996) on *M. croceipes* showed that increasing the number of odour experiences increases the accuracy of choosing the experienced odour. Others (Vet *et al.*, 1998) found that parasitoids can differentiate better between similar odours if they have associated a rewarding experience with one odour and an unrewarding experience with the alternative. Thus, future studies with a more developed training process and additional electrophysiological investigations can help further to explore and explain the discrimination-learning abilities in insect parasitoids.

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