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Parasitic Wasps Learn and Report Diverse Chemicals with Unique Conditionable Behaviors

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Abstract

Parasitoids exploit numerous chemical cues to locate hosts and food. Whether they detect and learn chemicals foreign to their natural history has not been explored. We show that the parasitoid *Microplitis croceipes* can associate, with food or hosts, widely different chemicals outside their natural foraging encounters. When learned chemicals are subsequently detected, this parasitoid manifests distinct behaviors characteristic with expectations of food or host, commensurate with prior training. This flexibility of parasitoids to rapidly link diverse chemicals to resource needs and subsequently report them with recognizable behaviors offers new insights into their foraging adaptability, and provides a model for further dissection of olfactory learning related processes.

Key words: behavior, learning, *Microplitis croceipes*, olfaction

Introduction

Historically, we have marveled at the capacity of organisms to sense chemical cues and learn to utilize them for foraging and other forms of biotic interactions (von Frisch, 1915, 1950; Muller-Schwarze and Silverman, 1980; Menzel, 1985; Vet *et al.*, 1995; Eisner and Meinwald, 1999). We have been able to harness the chemical detection ability in dogs for use in, for example, search and rescue, and detection of a multitude of illegal chemical substances. Despite the abundant information on olfaction and odor learning in organisms, the mechanisms or processes involved in the use of odor cues in biotic interactions are poorly understood (Alkasab *et al.*, 1999). Parasitoid species may provide good models for olfaction studies, because of their extraordinary capacity to utilize odors and learning to increase foraging success [reviews in (Vet *et al.*, 1995; Takasu and Lewis, 1996; DeMoraes *et al.*, 1998)].

Microplitis croceipes, a relatively specialized parasitoid of three highly polyphagous larval hosts, *Heliocoverpa zea*, *Heliothis virescens* and *Heliothis subflexa* (Lepidoptera: Noctidae) can detect their hosts through associative learning of hosts, host byproducts and host plant-related chemicals (Lewis and Tumlinson, 1988; Turlings *et al.*, 1989; DeMoraes *et al.*, 1998). This wasp species has also demonstrated the ability to learn and subsequently fly to the novel odors chocolate and vanilla, and these parasitoids can learn these odors in association with both food and hosts and subsequently respond in accordance to their physiological state (Takasu and Lewis, 1993). However, encounters with plant-related compounds, such as those associated with chocolate and vanilla, are expected within the scope of typical foraging activities.

To further explore the range of odors that female *M.croceipes* can detect and learn, they were conditioned to associate several structurally diverse chemicals that they would not encounter in their natural foraging. These compounds were cyclohexanone (a cyclic ketone), 3-octanone (an aliphatic ketone), octanal (an aliphatic aldehyde), diisopropyl aminoethanol (an aliphatic alcohol), and 2,4- and 3,4-dinitrotoluene (DNT) (aromatic hydrocarbons with methyl and nitrogen side chains). These compounds were presented with adult food as the unconditioned stimulus in flight bioassays. Behavioral responses to cyclohexanone for food-associated odor conditioned *Manduca sexta* (Daly *et al.*, 2001), and electroantennogram responses to cyclohexanone and diissopropyl aminoethanol for unconditioned male and female *M. croceipes* (Ochieng *et al.*, 2000; Park *et al.*, 2001) have been previously documented.

In addition to oriented flight, chemical cues also mediate a sequential array of parasitoid foraging behaviors, including hovering, landing, antennation and ovipositor probing (Lewis *et al.*, 1976). As the wasps get closer to the resource, their behavior is progressively more intense, distinctly characteristic and specific to the respective resource (Lewis *et al.*, 1976; Wäckers *et al.*, 2002). Thus, we also examined whether 'foreign' chemicals can mediate these characteristic close-range responses, which historically have been considered 'genetically fixed' to cues strongly associated with the subject resource.

Materials and methods

Larvae of *Heliocoverpa zea* Boddie (Lepidoptera: Noctuidae) were reared on pinto bean artificial diet (Burton, 1969). *Microplitis croceipes* were reared on *H.zea* larvae at 28°C, 60–70% relative humidity, and a 16 h/8 h light/dark cycle. All *M.croceipes* females were 2–4 days old when tested, and food-conditioned females had been provided water for 26–30 h at the time of the bioassays. A 50% sucrose solution by weight (99% pure) in distilled water was the unconditioned stimulus for all food conditioning, and feces from diet-fed third instar *H.zea* larvae was the unconditioned stimulus for hosts. Food conditioned females were fed for 5–10 s three times, with 30 s intervals between feeding (Takasu and Lewis, 1996). For conditioning, odors were delivered to females while they were feeding or antennating feces.

A structurally diverse group of compounds with very different volatilities was chosen to probe the limits of the wasps' behavioral responses. For this reason a variety of formulation methods were required to deliver the different odors to the wasps at constant rates. No attempt was made to determine the limits of responses to any of the compounds or to compare responses to equal quantities of different compounds by the wasps since this was beyond the objectives of this study. Cyclohexanone and diisopropyl aminoethanol, which are highly volatile, were formulated in capillaries (0.7 mm i.d.), and the length of the vapor column above the meniscus controlled release rate (Weatherston *et al.*, 1985). Both 2,4- and 3,4-DNT, which have low volatility, were dispensed from filter paper, and 3-octanone and octanal were dispensed from membrane formulations (Heath *et al.*, 1996). The release rate of each compound from its respective formulation was measured by collecting the volatized compound on a Super Q (Alltech, Deerfield, IL) filter trap and analyzing by capillary GC-MS (Heath *et al.*, 1993). All chemicals were obtained from Sigma and had chemical purity >99%.

Flight tests

Odor conditioning

Pure cyclohexanone and diisopropyl aminoethanol were loaded into 200 µl capillary tubes (diam. 1.68 mm) with one end sealed; the length of the vapor column above the meniscus was 1–2 mm. The capillary tube was held upright within a horizontal glass tube, and air was pushed through the larger tube at ~10 ml/min. Wasps fed while the odor was blown over their antennae. Control females fed while air was blown over their antennae. Females fed on sucrose and water at 2–3 mm from 3,4-DNT on filter paper (release rate ~11 ng/min). Control females were conditioned with sucrose and water only. Both control and test females fed on sucrose and water while exposed to membranes (Heath, 1996) loaded with pure 3-octanone (release rate 33 ng/min).

Test

The flight tunnel was previously described (Drost *et al.*, 1986). Females were placed on a stand (13 cm high), 80 cm downwind of the chemical source(s). The air speed in the wind tunnel was 60 cm/s. Pure cyclohexanone (300 ng/min) and diisopropyl aminoethanol (0.5 ng/min) were dispensed from capillary tubes placed vertically at the upwind end of the wind tunnel. Two filter papers (3 × 5 cm), folded in half with one loaded with yellow-colored 3,4-DNT (release rate ~11 ng/min) and the other a blank (filter paper only), were placed in the wind tunnel with the yellow-colored compound side of the paper facing away from the wasp to lessen any potential for visual attraction. Females were tested to membranes (Heath *et al.*, 1996) loaded with pure 3-octanone or octanal (33 ng/min).

Statistical analysis

For test and control females, the number of landings on the target or alternate source was recorded. Females that did not complete a flight after three chances or that did not take

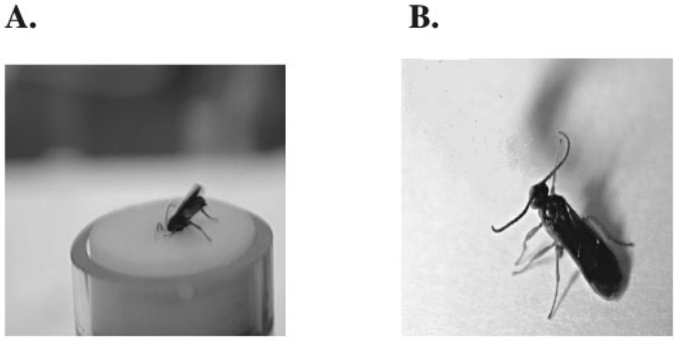

Figure 1 'Seeking' behavior of female *M.croceipes*; the odor-conditioned wasp enters a recessed hole where the conditioned odor emanates **(A)**, and 'coiling' behavior whereby females rise on their hind legs with a characteristic bending of their antennae **(B)**.

flight after 5 min were scored as no response. A total of 8–10 females per control and odor-conditioned treatments were assayed per day over 2- 4days. The influence of conditioned odor on the response of trained and control females were tested with chi-square analyses (SAS Institute, 1998).

We developed separate training protocols for the close-range food-associated and host-associated behaviors. The food-associated behavior that we termed ‘seeking’ is a modification of ‘area restricted search behavior’ and related food-specific behaviors (Curio, 1976; Wäckers *et al*., 2002). Instead of intense local search and substrate antennation, we trained the odor-conditioned wasp to enter a recessed hole from which the conditioned odor emanates (Figure 1A). The host-associated behavior we termed ‘coiling’ is the behavior this species exhibits just prior to host attack, whereby females rise on their hind legs with a characteristic bending of their antennae (Figure 1B).

Seeking behavior: food as unconditioned stimulus

Females were conditioned to 3-octanone (20 μl, 0.176 g/μl, dichloromethane) and 2,4-DNT (20 μl, 0.05 μg/μl, dichloromethane) on filter paper (2.3 cm diam.) placed in the center of a glass Petri dish (5 cm i.d., 1.5 cm high) covered with aluminum foil. A 4 mm^2 filter paper saturated with sucrose and water was placed on top and center of the aluminum foil and 9–10 holes (1 mm diam.) were punctured through the foil around the edge of the filter paper so that odors flowed upward and around the food source.

Test females were released near a recessed hole (2 mm diam) in the center of a hollowed out Teflon stand (external: 2 cm diam. × 1 cm high; internal: 1 cm i.d, 0.5 cm high). This stand was set directly on top of the compound-loaded filter paper. Females responded positively by entering the hole (at least half of their body length within the hole) within 10 s. Females responded negatively by walking over the hole and around the Teflon stand for >10 s.

Coiling behavior: host as unconditioned stimulus

Females antennated larval feces near 2,4-DNT or 3-octanone loaded on filter paper as for food conditioning. A piece of filter paper (0.7–1 cm diam.) loaded with 20 μl of 0.05 μg/μl 2,4-DNT with dichloromethane or 20 μl of 0.176 g/μl of 3-octanone with dichloromethane was presented to the wasp at the end of a pin (6 cm long). The paper was gently and continuously waved in front of the wasps while maintaining a distance of 0.5–1 cm; none of the females were allowed to touch the paper. Females responded positively by coiling within 10–30 s. Females responded negatively by showing no coiling response within 1 min.

Statistical analysis

For each odor, 3-octanone and 2,4-DNT, a total of 40 food and odor-conditioned females, 40 host and odor-conditioned females, 40 food-only conditioned females (control), and 40 host-only conditioned females (control) were tested for seek and coil behavioral responses. Positive and negative seeking and coiling behaviors were recorded for each female. For each odor, conditioning and testing was carried out over 4 days with a total of 10 females from each of the four conditioning regimes, five of which were tested for seeking and five tested for coiling responses. All females were conditioned and tested a single time. The influence of day and conditioning regime on the seeking and coiling behaviors of odor-conditioned and control females were tested with chi-square analyses (SAS Institute, 1998).

Results

There were no significant effects of the day of conditioning and testing on wasp responses for 3-octanone ($\chi^2_{\text{df3}} = 1.79$, $P = 0.615$, $n = 160$) and 2,4-DNT ($\chi^2_{\text{df3}} = 0.79$, $P = 0.851$, $n = 160$). Wasps that were trained to associate an odor with the food source were significantly more responsive to the learned chemicals in upwind flight tunnel bioassays than their control or untrained counterparts (Figure 2A–D). The trained wasps not only flew upwind more readily, but they showed more straightline or directed upwind flight compared to the controls, and they landed at the source of the learned chemical (Figure 2A–D).

After training with chemicals in association with either sugar water or host feces, the wasps successfully linked each of the chemicals to both the food and host resource as shown by significantly higher behavioral responses to each of the food- or host-associated odors as compared to untrained wasps (Figure 3A,B). We also found that wasps will express the specific behavior, seeking or coiling, exclusively dependent on the conditioned stimulus (Figure 3A,B).

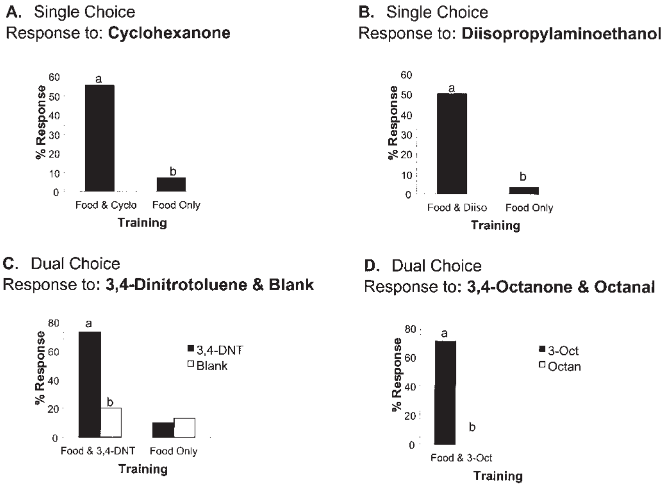

Figure 2 Flight and landing responses of female *Microplitis croceipes* to **(A)** cyclohexanone (= Cyclo at 300 ng/min, $n = 43$, $\chi^2_{\text{df1}} = 23.33$, $P = 0.000$), **(B)** diisopropylaminoethanol (= Diiso at 0.50 ng/min, $n = 38$, $\chi^2_{\text{df1}} = 21.99$, $P = 0.000$), **(C)** 3,4-dinitrotoluene (= 3,4-DNT at ~11 ng/min, and a blank = filter paper, $n = 20$, $\chi^2_{\text{df2}} = 13.72$, $P = 0.002$), **(D)** 3-octanone and octanal (= 3-Oct and Octanyl at 33 ng/min, $n = 20$, $\chi^2_{\text{df2}} = 26.67$, $P = 0.000$). Different letters above the bars are significantly different.

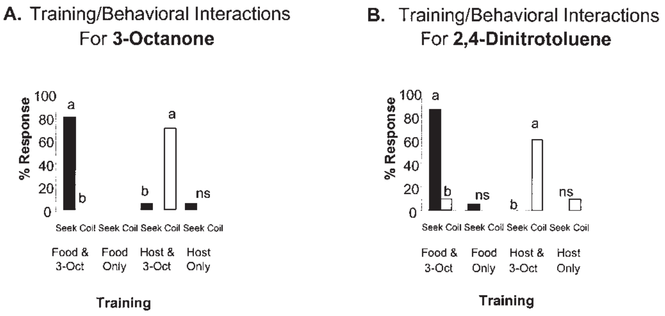

Figure 3 Seek and coil behaviors of female *Microplitis croceipes* conditioned with food and hosts and **(A)** 3-octanone and **(B)** 2,4-dinitrotoluene. Seek and coil responses to 3-octanone when trained with food and 3-octanone ($\chi^2_{\mathrm{df1}} = 26.67$, $P = 0.000$, $n = 40$), hosts and 3-octanone ($\chi^2_{\mathrm{df1}} = 15.82$, $P = 0.000$, $n = 40$) and hosts only ($\chi^2_{\mathrm{df1}} = 1.03$, $P = 0.311$, $n = 40$). Seek and coil responses to 2,4-DNT when trained with food and 2,4-DNT ($\chi^2_{\mathrm{df1}} = 22.57$, $P = 0.000$, $n = 40$), food only ($\chi^2_{\mathrm{df1}} = 1.03$, $P = 0.311$, $n = 40$), hosts and 2,4-DNT ($\chi^2_{\mathrm{df1}} = 15.17$, $P = 0.000$, $n = 40$) and hosts only ($\chi^2_{\mathrm{df1}} = 2.11$, $P = 0.147$, $n = 40$).

Discussion

Our study indicates that *M.croceipes* can learn a very broad range of chemicals as foraging cues, including chemicals not related to their natural history. The hosts of *M.croceipes* are highly polyphagous, feeding on >100 species of plants from several families (Li *et al.*, 1992). In addition, plants emit numerous volatiles with various functional groups and ranging in structure from short, straight carbon chains to aromatic hydrocarbons and complex multi-ring sesquiterpenes (Visser, 1979; Knudsen *et al.*, 1993; Paré and Tumlinson 1997). Thus, the natural volatile compounds that these insects are exposed to are many and diverse. Responses to such a diverse group of compounds suggest that their receptors are broadly tuned. Their ability to detect a broad array of chemicals and to show rapid adaptation, suggested by the speed with which they can be trained, may be related to their ecology, where they must locate very specific hosts species within highly variable and dynamic foraging environments.

We have successfully conditioned *M.croceipes* to many other compounds (Meiners *et al.*, 2002) (also J. Tomberlin *et al.*, unpublished data), but were not able to condition *M.crociepes* to nonanol and decanol (Meiners *et al.*, 2002), although their antennae will respond to these relatively long aliphatic alcohols (Li *et al.*, 1992). There are also differences in their responses to similar compounds that differ in the position or the type of the functional group (Meiners *et al.*, 2002); although they learned all compounds, they showed stronger responses to some of them. It may be possible to use both positive and negative rewards in conditioning to increase the responses to the lower affinity compounds (Vet *et al.*, 1998).

After training with chemicals in close association with either sugar water or host feces, the wasps successfully linked each of the chemicals to either the food and host resource. Untrained wasps did not show these behaviors. We also found that trained wasps will express the specific behavior, seeking or coiling, exclusively in accordance to the resource, food or hosts, associated with the conditioned stimulus. Thus, food as unconditioned stimuli will elicit seeking but not coiling behavior, whereas, host frass as unconditioned stimuli elicits coiling but not seeking behavior. In flight biaossays, Takasu and Lewis (Takasu and Lewis, 1993) found that *M.croceipes* could discriminate between expectations of food or hosts utilizing different odors associated with each resource. Future studies are planned to determine if they display their expectations with the more stereotyped behaviors of seeking and coiling. Our results indicate that, *M.croceipes* is highly versatile and effective in the mechanism by which they employ chemical cues to enhance foraging success. They are able to associate a highly diverse array of chemicals with the resource, as well as utilize the cue to determine resource specificity and assess its proximity.

This finding of additional levels of plasticity of predictable responses by parasitoids not only expands our understanding of their foraging mechanisms, but also opens important new prospects for innovative chemical detection technology (DARPA, 2002). The attribute of distinct behavioral displays in response to the learned chemicals provides observers a non-arbitrary means to monitor for positive detection of chemicals. These demonstrated abilities by insects, and probably other invertebrates, greatly expand our potential resources for biological detectors beyond those previously limited primarily to dogs. The short lifecycle and genetic diversity of these organisms, along with the apparent speed with which they can be trained, offers potential benefits of flexibility and convenience to the science of olfactory learning, and the science of biological detectors.

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References

Alkasab, T.K., Bozzo, T.C., Cleveland, T.A., Dorries, K.M, Pearce, T.C., White, J. and **Kauer, J.S.** (1999) *Characterizing complex chemosensors; information-theoretic analysis of olfactory systems.* Trends Neurosci., 22, 102–105.

Burton, R.L. (1969) *Mass rearing the corn earworm in the laboratory*. US Dept. Agric. Tech. Bull. ARS Ser., 33, 134.

Daly, K.C., Durtschi, M.L. and **Smith, B.H.** (2001) *Olfactory-based discrimination learning in the moth,* Manduca sexta. J. Insect Physiol., 47, 375–384.

DARPA (2002) Biosensor Technologies: Controlled Biological and Biomimetic Systems. Available at http://www.darpa.mil.dso.

Curio, E. (1976) The Ethiology of Predation. Springer-Verlag, Berlin.

DeMoraes, C.M., Lewis, W.J., Paré, P.W., Alborn, H.T. and **Tumlinson, J.H.** (1998) *Herbivore-infested plants selectively attract parasitoids*. Nature, 393, 570–573.

Drost, Y.C., Lewis, W.J., Zanen, P.O. and **Keller, M.A.** (1986) *Beneficial arthropod behavior mediated by airborne semiochemicals. I. Flight behavior and influence of preflight handling of* Microplitis croceipes *(Cresson).* J. Chem. Ecol., 12, 1247–1262.

Eisner, T. and **Meinwald, J.** (eds) (1999) Chemical Ecology: The Chemistry of Biotic Interaction. National Academy of Sciences. National Academy of Science Press, Wash. DC.

Heath, R.R., Epsky, N.D., Landolt, P.J. and **Sivinski, J.** (1993) *Development of attractants for monitoring carribean fruit flies (Diptera: Tephritidae)*. Fla. Entomol., 76, 233–244.

Heath, R.R., Epsky, N.D., Jimenez, A., Dueben, B.D., Landolt, P.J., Meyer, W.L., Aluja, M., Rizzo, J., Camino, M. and **Jeronimo, F.** (1996) *Improved pheromone-based trapping system to monitor* Toxotrypana curvicauda *(Diptera: Tephritidae)*. Fla. Entomol., 79, 38–48.

Knudsen, J.T., Tollsten, L. and **Bergstrom, L.G.** (1993) *Floral scents—a checklist of volatile compounds isolated by head-space techniques*. Phytochemistry, 33, 253–280.

Lewis, W.J. and **Tumlinson, J.H.** (1988) *Host detection by chemically mediated associative learning in a parasitic wasp*. Nature, 331, 257–259.

Lewis, W.J., Jones, R.L., Gross, H.R., Jr and **Nordlund, D.A.** (1976) *The role of Other behavioral chemicals in host finding by parasitic insects*. Behav. Biology, 16, 267–289.

Li, Y., Dickens, J.C. and **Steiner, W.W.M.** (1992) *Antennal olfactory responsiveness of* Microplitis croceipes *(Hymenoptera: Braconidae) to cotton plant volatiles*. J. Chem. Ecol., 18, 1761–1773.

Meiners, T., Wäckers, F. and **Lewis, W.J.** (2002) *The effect of molecular structure on olfactory discrimination by the parasitoid* Microplitis croceipes. Chem. Senses 27, 811–816.

Menzel R., (1985) *Learning in honeybees in an ecological and behavioral context.* In Hölldobler, B and Lindauer, M. (eds), Experimental Behavioral Ecology. Gustav Fischer, Stuttgart, pp. 55–74.

Muller-Schwarze, D. and **Silverman, R.M.** (1980) Chemical Signals: Vertebrates and Aquatic Invertebrates. Plenum, Washington, DC.

Ochieng, S.A., Park, K.C., Zhu, J.W. and **Baker, T.C**. (2000) *Functional morphology of antennal chemoreceptors of the parasitoid* Microplitis croceipes *(Hymenoptera: Braconidae).* Arthrop. Struct. Dev., 29, 231.

Paré, P.W. and **Tumlinson, J.H.** (1997) *De novo biosynthesis of volatiles induced by insect herbivory in cotton plants.* Plant Physiol., 114, 1161–1167.

Park, K.C., Zhu, J., Harris, J., Ochieng, S.A. and **Baker, T.C.** (2001) Electroantennogram responses of a parasitic wasp, *Microplitis croceipes*, to host-related volatile and anthropogenic compounds. Physiol. Entomol., 26, 69–77.

SAS Institute (1998) SAS User's Guide: Statistics. SAS Institute Inc., Cary, NC.

Takasu, K. and **Lewis, W.J.** (1993) *Host-and food-foraging of the parasitoid* Microplitis croceipes: *learning and physiological state effects*. Biol. Control, 3, 70–74.

Takasu, K. and **Lewis, W.J.** (1996) *The role of learning in adult food location by the Larval parasitoid,* Microplitis croceipes *(Hymenoptera: Braconidae)*. J. Insect Behav., 9, 265–281.

Turlings, T.C.J., Tumlinson, J.H., Lewis, W.J. and **Vet, L.E.M**. (1989) *Beneficial arthropod behaviour mediated by airborne semiochemicals. VII. Learning of host-related odours induced by a brief contact experience with host by-products in* Cotesia marginiventris *(Cresson).* J. Insect Behav., 2, 217–225.

Vet, L.E.M., Lewis, W.J. and **Cardé, R.T.** (1995) *Parasitoid foraging and learning.* In **Cardé, R.T., Bell, W.J**. (eds), Chemical Ecology of Insects, Vol. 2. Chapman & Hall, New York, pp. 65–101.

Vet, L.E.M., de Jong, A.G., Franchi, E. and **Papaj, D.R.** (1998) *The effect of complete versus incomplete information on odor discrimination in a parasitic wasp.* Anim. Behav., 55, 1271–1279.

Visser, J.H. (1979) *Electroantennogram responses of the Colorado beetle* (Leptinotarsa decemlineata), *to plant volatiles*. Entomol. Exp. Appl., 25, 86–97.

von Frisch, K. (1915) *Der Farbensinn und formensinn der biene.* Zool. Abt. Allg. Zool. Physiol., 35, 1–182.

von Frisch, K. (1950) Bees, Their Vision, Chemical Senses and Language. Great Seal Books, Cornell Univeristy Press, Ithaca, NY.

Wäckers, F.L., Bonifay, C. and **Lewis, W.J.** (2002) *Conditioning of appetitive behavior in the Hymenopteran parasitoid* Microplitis croceipes*.* Entomol. Exp. Appl., 103, 135–138.

Weatherston, I., Miller, D. and **Dohse, L.** (1985) *Capillaries as controlled release devices for insect pheromones and other volatile substances—a reevaluation. Part I. Kinetics and development of predictive model for glass capillaries*. J. Chem. Ecol., 11, 953–965.

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