# Associative Learning of Complex Odours in Parasitoid Host Location

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## Abstract

In this paper we address the question how hymenopteran parasitoids deal with complex odour bouquets, using *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) as a model. We examined the capacity of *Microplitis croceipes* to respond to individual compounds in flight chamber experiments after conditioning parasitoids with a mixture consisting of 2-octanone, methyl jasmonate and β-caryophyllene. Parasitoids were given a choice between single compounds from the training mixture and β-ocimene as an unrewarded alternative. When compared with control individuals lacking experience with the odour mixture, parasitoids trained to the odour blend showed an increased preference for 2-octanone and β-caryophyllene, but not for methyl jasmonate. However, when trained with methyl jasmonate alone, parasitoids were able to respond to this compound. This demonstrates that parasitoids can learn to respond to individual compounds following experience with an odour mixture. However, for certain compounds of a mixture, learning can be blocked by other mixture components. Further experiments in which parasitoids were conditioned and challenged with two compound mixes confirmed that the olfactory background can affect recognition of individual compounds.

**Key words**: background odour, complex blends, insect learning, mixture recognition, plant volatiles

## Introduction

Volatile semiochemicals play a key role in the host location process of parasitoids [reviewed by Vinson (Vinson, 1991)] and in their foraging for sugar sources (Wäckers, 1994). Host searching parasitoids respond especially to semiochemicals released by herbivore-infested plants. They can use a range of host- or plant-derived volatiles during host location (Rutledge, 1996; Turlings and Benrey, 1998). In many plants, over 100 volatile plant components have been identified (Visser, 1979; Etiévant *et al.*, 1984; Borg-Karlson and Tengö, 1986; Knudsen *et al.*, 1993; Blight *et al.*, 1997). The quality and quantity of these blends can fluctuate according to abiotic conditions (Robacker *et al.*, 1982), inherent factors such as phenology (Hedin, 1976), or external biotic factors such as type, quantity and time of herbivore plant damage (Turlings *et al.*, 1998), and even oviposition (Hilker *et al.*, 2002). Therefore natural odours encountered by parasitoids in their natural environment are both complex and variable mixtures.

Associative learning allows parasitoids to focus on the most reliable cues (Vet *et al.*, 1990; De Jong and Kaiser, 1991; Papaj and Lewis, 1993). It has also been shown that parasitoids can learn a wide range of volatiles, including ecologically relevant (Vet and Groenwold, 1990; Kester and

Barbosa, 1991) as well as novel odours (Lewis and Takasu, 1990).

Despite the broad range of studies on parasitoid learning, there are very few studies that examined the perception of defined blends in parasitoids (Fukushima *et al.*, 2002). Studies on parasitoid learning have typically trained parasitoids to essential oils or pure chemicals. However, the parasitoid *Leptopilina heterotoma* was shown to learn to respond to single C6-compounds in a complex background of yeast odour (Vet and Groenwold, 1990; Vet *et al.*, 1998).

To study the learning mechanisms that are required for the identification of defined blends we used the braconid wasp *Microplitis croceipes*, as a model system. *Microplitis* has been successfully used for the study of various learning paradigms (Lewis and Takasu, 1990; Wäckers *et al.*, 2002) and we have recently started to survey the effect of molecular structure of volatiles on its olfactory discrimination (Meiners *et al.*, 2002). Furthermore, the larvae of its noctuid host species, *Helicoverpa* and *Helitothis*, are polyphagous (Fitt, 1989) and the parasitoid is challenged during host location with a multitude of blends with different components.

The odours selected for training and testing the wasps

were 2-octanone, β-caryophyllene and methyl jasmonate. β-Ocimene was used as an untrained control odour in subsequent dual choice tests. All compounds, with the exception of 2-octanone, are plant volatiles that play an important role in plant defence and could signal parasitoids the presence of wounded plants (McCall *et al.*, 1993; Takabayashi *et al.*, 1994; Turlings *et al.*, 1998). Thus, these compounds may reflect odours that are important within the host-searching realm of *M. croceipes*. 2-Octanone is a ketone known from cheese odour and serves as an attractant for cheese mites (Yoshizawa *et al.*, 1970). This volatile was chosen, as it has no known ecological function for *M. croceipes*.

The parasitoids were trained and challenged with either a single component or blends of two and three components. These experiments, albeit with a limited number of compounds, are a crucial first step in understanding how odour learning takes place under more natural situations, in which biologically relevant odours occur in blends and against a background of additional olfactory stimuli.

## Materials and methods

### Insects

*Heliothis zea* was reared as described by Burton (1969) at 28°C, 50–70% relative humidity (RH), and a 16 h light: 8 h dark photoperiod. *Microplitis croceipes* was reared on *H. zea* larvae according to the method of Lewis and Burton (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 the one described previously (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

Female *M. croceipes* were allowed to contact fresh frass (~20 mg) of artificial diet-fed *H. zea* larvae offered 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, 1 µl of the given solution was applied on 0.5 × 0.5 cm filter paper, which was subsequently placed in a glass pipette. While the female 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. As a control parasitoids were trained to clean air only using a pipette with a blank piece of filter paper. Clean air (and not the solvent) was used as

control odour during training since it could not be excluded that the parasitoids are able to learn to respond to the solvent itself.

### Chemicals

The following chemicals were used as training odours: (i) a mixture of 2-octanone, β-caryophyllene and methyl jasmonate (ternary odour mixture); (ii) a mixture of β-caryophyllene and methyl jasmonate (binary mixture); and (iii) methyl jasmonate alone. β-Ocimene was used as unconditioned control odour during testing. Compounds were used as 5% (vol/vol) solutions in hexane–paraffin. All tested compounds were purchased from Sigma (St Louis, MO). They were at least 95% pure except for β-ocimene, which consisted of isomers (~70% (*E*)- and 30% (*Z*)-β-ocimene).

### Testing

Fifteen minutes after conditioning, the female wasps were introduced into a flight chamber using a 7.6 ml shell vial at a position 80 cm downwind of two odour sources. The two odours were presented on strips of filter paper (1 × 2 cm) on which 1 µl of the respective solution had been placed. Both filter papers were attached to a glass pipette placed vertically on a stand and spaced 12 cm apart.

Each wasp 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'.

Parasitoids trained to the ternary odour mixture were offered a choice between the ternary blend and β-ocimene (both diluted to 5% with hexane–paraffin). Next, individual females trained to the odour blend were given the choice between each single compound of the conditioned ternary odour mixture and the unconditioned odour (β-ocimene). The response of the wasps to methyl jasmonate and β-ocimene was investigated in a dual choice test after the parasitoids had been trained to methyl jasmonate alone. A control group that had been trained with frass in the presence of pure air was also tested in each of the above experiments.

Finally the preference of females trained to the binary mixture, the ternary mixture or control females (trained to pure air) was tested when given a choice between the binary mixture and the mixture of the three compounds.

For each different treatment in the wind tunnel experiments five females were tested every day for 5 days (a total of 25 females for each treatment). The range of non-responders during the test period of 13 days was 10–40%. On average, 30% of the wasps did not respond.

Differences in the total number of wasps that completed a flight to one odour alternative within each two-choice experiment were analysed by $\chi^2$ statistics. Whenever the difference was found to be significant, we concluded that a

preference for the more frequent visited odour exists. Differences between the wasps' responses to the two odour sources over two different treatments were also compared by $\chi^2$ statistics. Whenever the difference was found to be significant, we concluded that learning had occurred.

## Results

### Learning of single components in a mixture

Parasitoids trained to control air showed a non-significant preference towards the mixture of 2-octanone, β-caryophyllene and methyl jasmonate when it was pitted against the untrained odour β-ocimene in the flight chamber. Following experience with the ternary mixture, more parasitoids completed their flight and landed significantly more often on the filter paper treated with the mixture than on the control filter paper (Figure 1). However, no learning occurred after parasitoids had been trained with the ternary odour mixture.

Parasitoids trained to control air tended to have a non-significant innate preference for the plant odour β-ocimene over 2-octanone (Figure 2a). They showed no difference in their flight response to the plant odours β-ocimene and β-caryophyllene (Figure 2b) or β-ocimene and methyl jasmonate (Figure 2c) in dual choice tests.

Female *M. croceipes* trained to the ternary mixture successfully learned to respond to 2-octanone (Figure 2a) and also to caryophyllene (Figure 2b) when these compounds were pitted against the untrained β-ocimene. In contrast, the female wasps experienced with the mixture did not prefer methyl jasmonate to the control odour (Figure 2c). However, when they were trained to methyl jasmonate alone they successfully responded to that compound (Figure 3).

### Learning of complex odours

Females of *M. croceipes* trained to air showed no prefer-

ence for the binary mixture of methyl jasmonate and caryophyllene lacking 2-octanone or the ternary odour blend in dual choice tests (Figure 4). This confirms the lack of an innate preference for 2-octanone (Figure 2a). Also females trained to the two components methyl jasmonate and caryophyllene alone did not discriminate between the binary mixture and the ternary odour blend (Figure 4). When 2-octanone was used together with the other two components to train the parasitoids, the wasps showed a non-significant tendency to prefer this previously experienced blend over the blend of methyl jasmonate and caryophyllene. Despite this apparent tendency, no learning
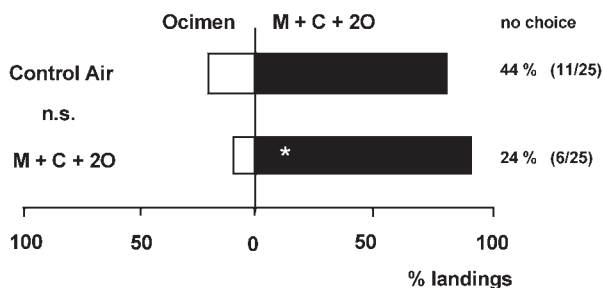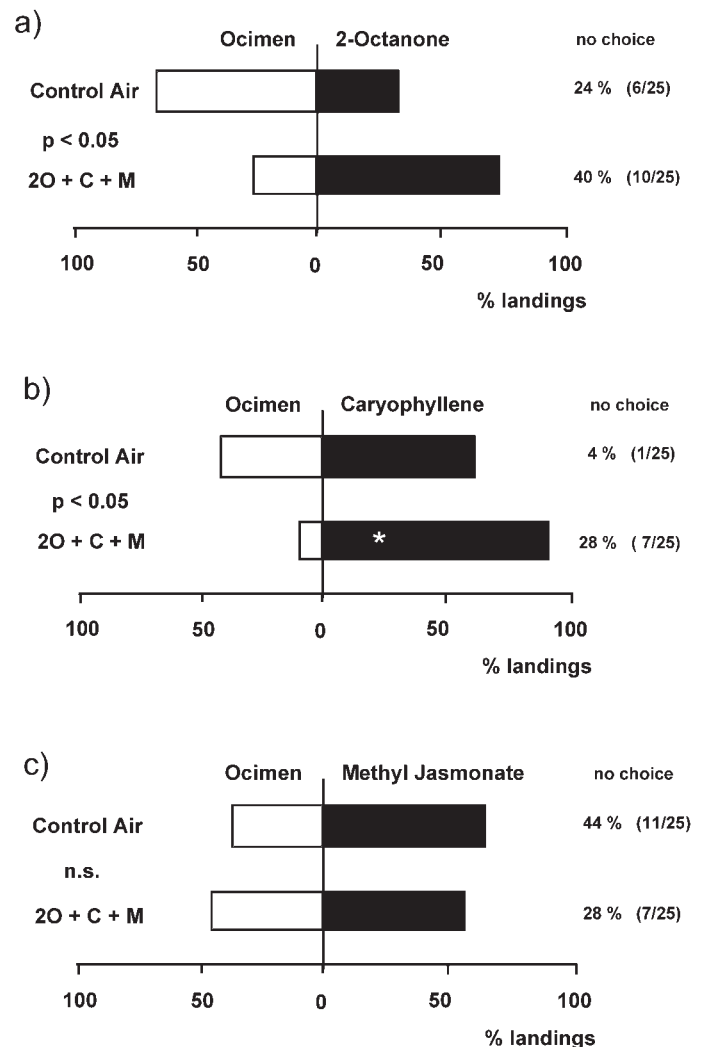
**Figure 1** Responses of *M. croceipes* females trained to air only (*n* = 25) or a ternary mix of 2-octanone (2O), β-caryophyllene (C) and methyl jasmonate (M) (*n* = 25) in flight chamber choice experiments. Bars indicate the percentage of completed flights to the mix (black bars) or to β-ocimene (white bars). Statistical testing was performed by $\chi^2$ tests. Asterisk indicates a significant difference in the choice between two odour sources (*P < 0.05). No significant difference between different choice tests is given (n.s. not significant).

**Figure 2** Responses of *M. croceipes* females trained to air only or a ternary mix of 2-octanone (2O), β-caryophyllene (C) and methyl jasmonate (M) in flight chamber choice experiments. Bars indicate the percentage of completed flights of 25 females to **(a)** 2-octanone (black bars) or β-ocimene (white bars), **(b)** β-caryophyllene (black bars) or β-ocimene (white bars), and **(c)** methyl jasmonate (black bars) or β-ocimene (white bars). Statistical testing was perfomed by $\chi^2$ tests. Asterisk indicates a significant difference in the choice between two odour sources (*P < 0.05). Significant difference between different choice tests is given (n.s. not significant).
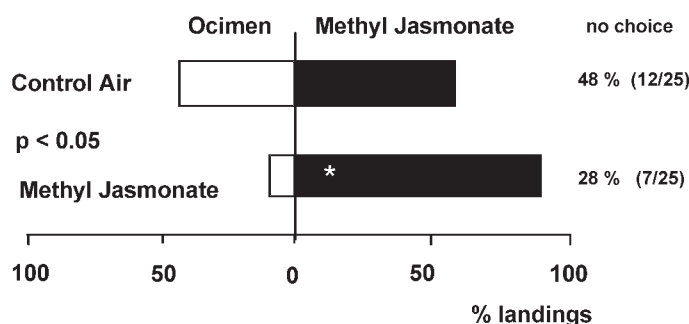
**Figure 3**  Responses of *M. croceipes* females trained to air only (*n* = 25) or methyl jasmonate (*n* = 25) in flight chamber choice experiments. Bars indicate the percentage of completed flights to methyl jasmonate (black bars) or β-ocimene (white bars). Statistical testing was performed by χ² tests. Asterisk indicates a significant difference in the choice between two odour sources (*P* < 0.05). Significant difference between different choice tests is given.
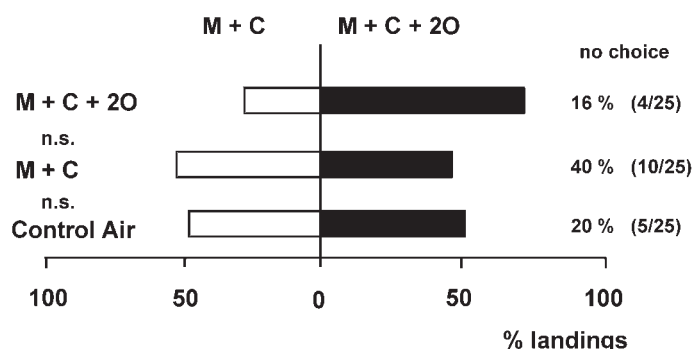


**Figure 4**  Responses of *M. croceipes* females trained to a ternary mix of 2-octanone (2O), β-caryophyllene (C) and methyl jasmonate (M), to a binary mix of β-caryophyllene (C) and methyl jasmonate (M), and to air only in flight chamber choice experiments. Bars indicate the percentage of completed flights of 25 females each to the binary mix (white bars) or the ternary mix (black bars). No significant differences between different choice tests or between the two odour sources were detected (χ² tests, *P* > 0.05, n.s. not significant).

occurred after training the wasps with the two- and three compound mixes.

## Discussion

### Learning of single components in a mixture

Our results show that *M. croceipes* are able to learn to respond to single compounds within odour blends. The wasp appears to prefer β-caryophyllene and 2-octanone over the untrained stimulus β-ocimene after experiencing the ternary mixture. Such a learning process was not found in the case of methyl jasmonate. However, wasps conditioned to methyl jasmonate alone did show a significant increased preference for this compound over β-ocimene. This indicates that the two other compounds affected the learning of methyl jasmonate during training. Volatility differences

could have influenced the learning abilities of *M. croceipes*. However, Laloi *et al.* (Laloi *et al.*, 1999) showed that concentration differences of conditioned stimuli from 10 ng to 1 µg had no relevant effect on the learning of blends in honey bees. Furthermore, Park *et al.* (Park *et al.*, 2001) showed in electroantennogram studies that the antenna of *M. croceipes* is more sensitive to less volatile compounds (e.g. methyl jasmonate, β-caryophyllene) than to more volatile compounds (e.g. β-ocimene). Thus a lower volatility might be compensated by a higher sensitivity.

Our results are consistent with studies of mixtures of chemical stimuli that demonstrate interactions between the odours perceived in mixes in a broad array of animals (Schiet and Cain, 1990; Akers and Getz, 1993; Laska and Hudson, 1993; Patterson *et al.*, 1993; Derby *et al.*, 1996; Smith 1998). Sensory receptors for non-pheromonal odorants can respond to many compounds and exhibit complex interactions in response to compounds in mixtures (Ache, 1989; Atema *et al.*, 1989). Mixture suppression is a common result in recordings of sensory cell responses to pure compounds and to mixtures (Ache, 1989; Akers and Getz, 1993) and might explain that interactions in some mixtures are stronger than in others.

Consistent with the data obtained from mixture recognition studies in honey bees (Pham-Delégue *et al.*, 1993; Wadhams *et al.*, 1994; Laloi *et al.*, 2000), our wind tunnel studies confirm a hierarchy within the components of a mixture, with some components eliciting mixture recognition better than others. Laloi *et al.* (Laloi *et al.*, 1999) found that individual honey bees differed in their learning ability. Good learners perceived a hierarchy among the components of a mixture, with some compounds being more representative of the conditioning mixture than others.

### Learning of complex odours

Wasps trained to air only, to the binary mixture of methyl jasmonate and β-caryophyllene, and to the ternary mixture containing additionally 2-octanone did not prefer one of the odour mixes in the flight chamber (Figure 4). This indicates that wasps trained to the binary mixture treated 2-octanone as a neutral background odour. Although females of *M. croceipes* conditioned to the ternary mixture responded to 2-octanone when offered alone (see above), they did not prefer this compound in the background of the two other trained compounds. Laloi *et al.* (Laloi *et al.*, 2000) found a similar result in honey bees, which recognized components from a blend in no-choice tests, though they were less active in choice tests with free-flying bees. Furthermore, they showed that the discrimination of the individual compounds of six component mixtures in honey bees is affected by the other components of the mixture. It is believed that a small number of key compounds account for a major part of the behavioural activity of the total mixture in honey bees (Blight *et al.*, 1997). Smith and Cobey (Smith and Cobey, 1994) compared response levels in honey bee proboscis

extension with binary mixtures using citral, geraniol, hexanal and 1-hexanol. Three out of four binary mixtures caused no significant difference in response levels to mixtures compared with responses to the components. However, after conditioning to a hexanal/1-hexanol mixture the response level to 1-hexanol was significantly lower than to hexanal and the mixture.

In many cases it might be favourable for a parasitoid to generalize very similar odours and not to invest too much time or sensory–physiological effort to differentiate between several odours that might be promising indicators of hosts in natural environments. Vet *et al.* (Vet *et al.*, 1998) found that *Leptopilinia heterotoma* can adjust its degree of discrimination between similar odours (e.g. apple varieties) according to the profitability of the information in terms of host encounters that is connected with the odour. Coupling a rewarding experience with one odour and an unrewarding experience with the other odour allowed the parasitoids to differentiate between the odours. A further developed training process might help to enhance the accuracy of conditioning and testing of *M. croceipes*. Further detailed studies are required to investigate the possible correlations between the initial level of activity of compounds and their significance in mixture recognition, as well as the interactions between compounds.

## Acknowledgements

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