

Associative odour learning affects mating behaviour in *Aphidius ervi* males (Hymenoptera: Braconidae)

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Abstract. We used the aphid parasitoid *Aphidius ervi* reared in its host *Acyrtosiphon pisum* to examine if male sexual attractive responses can be conditioned to an odour (vanilla) that is not present in the natural environment. We used prior mating experience (exposure to females) as a non-conditioning stimulus and vanilla odour as a conditioning stimulus. The behavioural responses were tested in a glass Y-olfactometer just after eclosion (i.e., initial response) and after a training experience (i.e., trained response). During the 10-min training period individual males were allowed to copulate with a virgin female with or without vanilla odour present, or were exposed only to vanilla odour. Wing fanning was a recurrent behaviour which denoted increased sexual attraction to a volatile stimulus. Total time and time doing wing fanning in each olfactometer arm were determined. Vanilla odour, which initially did not elicit sexual-related behaviours, triggered strong sexual attractive responses when males were trained to females plus vanilla odour. Neither copulation only nor vanilla odour only treatments elicited such behaviours in trained males. The results are discussed in terms of parasitoid learning ability and its ecological consequences.

INTRODUCTION

Learning allows female parasitoids to display plastic responses in changing environments while searching for oviposition sites (Turlings et al., 1993). In spite of its relevance, no solid evidence has been published in relation to the occurrence of learning in males searching for females. Females are prone to copulate during a brief period of time, approximately 8 h after eclosion (Mackauer, 1969). After this period, they tend to refuse males and spend most of their time searching for an oviposition site. In contrast, males search for females for almost their entire adult lifetime.

We addressed the question if males of the parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) are able to learn by association during copulation, and respond to novel stimuli not present in their natural environment. We hypothesise that males learn to respond to a training stimulus (e.g., vanilla odour) present in the mating environment, and we propose that this learning is mainly associative.

METHODS

Olfactometer

Y-tube olfactometers were used to investigate the responses of *A. ervi* males when presented with a choice of odours. The olfactometers used (9 cm-long tube and 6 cm-long arms) allowed the parasitoids to explore both arms and to move from one arm to the other. Olfactometers were changed after each test and cleaned successively with ethanol, odour-free soap, and distilled water. Pure air to feed into the stimulus chambers was obtained from an ultrapure synthetic air cylinder (Indura®). The air flow during the tests was maintained at 250 ml/min, light intensity at 3600 lux, and temperature at 20°C. Experimental parasitoids were exposed to air only, or to air with commercial vanilla odour (Marcopolo®) at a concentration of 0.01 µl vanilla/ml air.

Insects

Parasitoids were obtained from a laboratory culture maintained on *Acyrtosiphon pisum* Harris on broad bean (*Vicia faba*

L.). Mummies were isolated in Petri dishes containing a wet piece of filter paper, and newly eclosed male and female insects were fed with water only.

In order to assess changes in behavioural responses due to experience, males were tested twice with the same stimulus set. The first time the males were tested within 8 h of eclosion and before any induced experience (initial response, IR); the second time, they were tested 30 min after being subjected to a training protocol (trained response, TR). Each test lasted 5 min, measured from the time a male was introduced in the olfactometer. The tests were videorecorded.

Training protocol

After the recording of the initial responses, male parasitoids were randomly assigned to three groups that were subjected to different training protocols. Males were exposed to stimuli in a Petri dish during 10 min; this experience was followed by 30 min in an empty Petri dish before testing the trained response.

In the first group, males were introduced into a Petri dish with a virgin female (within 8 h of eclosion) in the presence of a vanilla air flow at the same concentration and flux as used in the Y-tube (n = 13). In the second group, only a female was present in the Petri dish (n = 18). In the third group (n = 16), males only experienced the vanilla stimulation. In the first two protocols, males copulated with the female within a few minutes. The first training protocol is associative while the other two are non-associative learning controls. If males are capable of learning by association during copulation, they are expected to change their response to the vanilla stimulus alone.

Behaviour

As measures of the attraction to a stimulus, time spent in each arm of the Y-olfactometer, and time spend wing fanning were recorded. This behaviour is related to male attraction to the females and is an important behavioural display during courtship (Mackauer, 1969; Battaglia et al., 2002). Behavioural data were collected utilizing the JWatcher Software 0.9® (Blumstein et al., 2000).

TABLE 1. Wing fanning responses between female plus vanilla (F + V), female only (F), and vanilla only (V) treatments: negative values represent a vanilla-biased behavioural display, while positive values represent a stronger response to the pure air control arm. In A, the mean value for each treatment in initial and trained responses is shown; in B, MANOVA and Post hoc LSD test results are displayed.

A: Experimental results									
Wing Fanning Response	Mean	SD	n	Mean	SD	n	Mean	SD	n
	F + V			F			V		
Initial	-0.0027	0.0061	13	0.0007	0.0024	18	-0.0002	0.0008	16
Trained	0.0051	0.0062	13	-0.0006	0.0023	18	-0.0016	0.0061	16

B: MANOVA fixed effects for Trained Response – Initial Response, $F(2 \text{ d.f.}) = 9.3218$, $p = 0.0028$. Post hoc LSD test (P values shown)

Wing Fanning	F + V	F	V
F + V	–	0.004*	0.0006*
F	–	–	0.9865
V	–	–	–

Analysis

In order to combine measurements in the olfactometer in a single variable, the differences between the proportions of time spent in each arm and between wing fanning activity in each arm were calculated as (time in vanilla arm/total time in olfactometer) – (time in air only arm/total time in olfactometer). Hence, attraction responses to vanilla stimulus were represented with positive values while attractive responses to air only appeared as negative values. In order to evaluate changes in behavioural responses in relation to the different training protocols, a two-way repeated measures ANOVA was used with treatment as one factor and temporal changes in the response as measures. Differences between treatments, evaluated as the difference between TR and IR, were analysed with a fixed effects MANOVA and post hoc LSD tests for planned comparisons. Data satisfied the assumptions of the tests employed.

RESULTS

There were no statistically significant differences in the total time in each arm neither in temporal changes, between treatments or their interaction. A significant increase of the time devoted to wing fanning was observed after female plus vanilla treatment [repeated measures ANOVA: treatment \times measure interaction, F -Ratio (2 d.f.) = 9.03, $p = 0.0005$], while no differences were observed for the vanilla-only or female-only treatments. This last result is relevant because wing fanning is an exclusively courtship display and its appearance in response to the vanilla-only stimulus is clear evidence of the acquisition of a conditioned response.

Comparisons between treatments of the TR-IR measures of time spent wing fanning showed significant differences [fixed effects MANOVA : $F(2 \text{ d.f.}) = 9.3218$ $p = 0.0028$], post hoc LSD test showing that the female plus vanilla treatment was statistically different from female only and vanilla only treatments (Table 1).

DISCUSSION

Our results demonstrate associative learning capacity in relation to an artificial stimulus in *A. ervi* males when the stimulus is experienced during copulation. For vertebrates, and some invertebrates, copulation experience affects the subsequent behaviour of males, improving their copulatory skills and levels of activity. In rats, copulation with females in the presence of cadaverine essence, can change trained male responses to cadaverine from repulsion to attraction and sexual arousal, in a phenomenon which has been called Conditioned Place Prefer-

ence (CPP) (Pfaus et al., 2001). To the best of our knowledge, this is the first evidence of CPP for a parasitic wasp and possibly for a holometabolous insect, and demonstrates that insect males can utilize secondary cues learned during copulatory experience, over those derived directly from the female, such as pheromones.

Previously, we demonstrated that the olfactometric response of female and male *A. ervi* parasitoids to volatiles from the host-plant depended on their previous rearing experience (Daza-Bustamante et al., 2002). It was further shown that oviposition environment also affected the attraction of females to plant volatiles (Du et al., 1997; Rodriguez et al., 2002). The present evidence constitutes a good framework to test the influence of the host-plant system on mate searching, and the role of learning in the maintenance of parasitoid-host specificity systems (Bush, 1975; Tremblay & Pennacchio, 1988; Digilio & Pennacchio, 1992).

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