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Associative learning of plant odorants activating the same or different receptor neurones in the moth *Heliothis virescens*

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Summary

The importance of olfactory learning in host plant selection is well demonstrated in insects, including the heliothine moths. In the present study olfactory conditioning of the proboscis extension response was performed to determine the moths' ability to learn and discriminate three plant odorants: β-ocimene and β-myrcene (activating the same receptor neurone type), and *racemic* linalool (activating two different types). The conditioned stimulus (CS) was an air puff with each odorant blown into a constant air stream and over the antennae, and the unconditioned stimulus (US) was sucrose solution applied first to the antennal taste sensilla, then to the proboscis. Conditioning with increasing odorant concentrations induced increased learning performance. The concentration threshold for learning was 100 times lower for *racemic* linalool than for the two other odorants, a fact that can be correlated with a higher sensitivity of the moths' antennae to *racemic* linalool as shown in electroantennogram recordings. After correcting for the different odour sensitivities, the moths' ability to discriminate the odorants was studied. Differential conditioning experiments were carried out, in which moths had to distinguish between a rewarded (CS+) odorant and an explicitly unrewarded odorant (CS–), choosing odour concentrations giving the same learning rate in previous experiments. The best discrimination was found with β-myrcene as the rewarded odorant and *racemic* linalool as the unrewarded. The opposite combination gave lower discrimination, indicating a higher salience for β-myrcene than for *racemic* linalool. The moths could also discriminate between β-ocimene and β-myrcene, which was surprising, since they activate the same receptor neurone type. No difference in salience was found between these two odorants.

Key words: olfactory learning, linalool, ocimene, myrcene, proboscis extension response, differential conditioning, primary odorant, recordings.

Introduction

Apart from its well-known dedication to intra- and inter-specific communication, the olfactory system of herbivorous insects has also evolved for perceiving plant odour information, which is crucial for finding suitable hosts that provide food, a place for meeting mates and a substrate for oviposition. This sensory system possesses not only an innate specialisation for recognising certain odorants, but can also be modulated by experience with the plants. This kind of modulation involved in olfactory learning seems particularly relevant in polyphagous insects using a broad range of host plants or in social insects, like the honeybee (*Apis mellifera*) that chooses flowers among many available species at any given time. Most of the knowledge gathered up to the present on insects' olfactory learning abilities involved feeding (appetitive learning) and the underlying mechanisms, and came from studies on the honeybee, by the use of the proboscis extension response (PER) in classical conditioning (Bitterman et al., 1983; Menzel, 2001; Menzel and Müller, 1996). Stimulation of the taste sensilla on the honeybee's antenna with sucrose (unconditioned stimulus, US) elicits extension of the proboscis, whereas stimulation with an odour (conditioned stimulus, CS) prior to conditioning only rarely elicits proboscis extension. However, after explicitly pairing the odour and sucrose stimulations, the honeybee will develop a conditioned PER to the odour alone (conditioned response, CR). Multiple learning trials lead to a high, stable and long-lasting memory (>4 days) in the honeybee (Menzel, 1999). Appetitive olfactory conditioning has also been demonstrated in several other insect species, like the bumblebee (*Bombus terrestris*) and moths (*Heliothis virescens, Manduca sexta* and *Spodoptera littoralis*)

(Daly and Smith, 2000; Fan and Hansson, 2001; Fan et al., 1997; Hartlieb, 1996; Hartlieb et al., 1999; Hartlieb and Hansson, 1999; Laloi et al., 1999). For a variety of phytophagous insects, field experiments have underlined the importance of olfactory learning in host selection (Papaj and Prokopy, 1989), including in the heliothine moth *Helicoverpa armigera* (Cunningham et al., 1998a,b,1999). Previous experience with a flowering host species seemed to increase the probability of subsequent selection of that species for nectar foraging as well as for oviposition. In accordance with this, wind tunnel experiments have shown that moths preferred odours previously paired with a sucrose reward (Cunningham et al., 2004).

A range of neurophysiological methods in the honeybee and in the fruitfly *Drosophila melanogaster* have allowed characterisation of the brain structures involved in olfactory learning and memory (Heisenberg, 2003; Menzel, 2001). In the honeybee, multiple convergence sites between the olfactory (CS) and the taste (US) pathway have been identified. Thus, a ventral unpaired median neurone (VUMmx1) was found, the depolarisation of which was shown to substitute for the rewarding part of the US in associative learning experiment (Hammer, 1993). This neurone has dendrites and its cell soma in the taste centre, the suboesophageal ganglion, and has extensive arborisations in three olfactory areas: the antennal lobe, the lateral protocerebrum and the calyces of the mushroom bodies. Several studies using optical or intracellular recordings have shown changes of odour responses in the antennal lobe and in the mushroom bodies after olfactory learning (Faber et al., 1999; Faber and Menzel, 2001; Mauelshagen, 1993; Sandoz et al., 2003; Daly et al., 2004; Yu et al., 2004). At the peripheral level, possible changes of odour responses by antennal receptor neurones (RNs) have been discussed in studies that have given contradictory results (De Jong and Pham-Delègue, 1991; Pham-Delègue et al., 1997; Sandoz et al., 2001; Vet et al., 1990; Wadhams et al., 1994). In most of these studies techniques have been used that recorded summated peripheral responses or processed responses in higher-order neurones, making it difficult to understand where the changes occur. Heliothine moths, for which the olfactory RNs have been well described, represent an interesting model for evaluating possible changes in odorant responses through learning. However, before the search for learning-induced changes can begin, a particular effort must first be made to describe better the learning mechanisms in these species. The present study represents such an effort in *H. virescens*, focusing on three plant odorants with a strong biological relevance for this moth.

By the use of gas chromatography linked to electrophysiological recordings from single RNs, 19 types of plant odour RNs have been classified in this species (Mustaparta, 2002; Røstelien et al., 2000a,b; Stranden et al., 2003a,b; T. Røstelien, M.S., A.-K. Borg-Karlson and H.M., unpublished). The RNs are characterised by strong responses to one odorant (primary odorant) and weak responses to a few others with related molecular structures (secondary odorants). Among them are three frequently occurring RN types, tuned to *E*-β-ocimene (3*E*-3,7-dimethyl-1,3,6-octatriene), geraniol (2*E*-3,7-dimethyl-2,6-octadien-1-ol) and (*S*)-(+)-linalool (3,7-dimethyl-1,6-octadien-3-ol), respectively. β-Myrcene (7-methyl-3-methylene-1,6-octadiene) and *Z*-β-ocimene (3*Z*-3,7-dimethyl-1,3,6-octatriene) are secondary odorants of the *E*-β-ocimene RN type, (R)-(–)-linalool of the (*S*)-(+)-linalool type, and both enantiomers of linalool are secondary odorants of the geraniol type.

In the first part of this study, parameters of the learning procedure affecting the acquisition of the CS–US association were tested. Male and female moths were subjected to acquisition procedures with increasing concentrations of *racemic* linalool, a floral volatile compound activating at least two RN types. As the time parameters of CS and US presentations are critical for learning, we also studied the influence on acquisition of the interval between CS and US onset, i.e. the inter-stimulus interval (ISI). We then asked whether odorants with differential activation of RN types cause different learning rates, a fact that would point to differences in their salience. As β-myrcene activates the same RN type as *E*-β-ocimene, but with a lower efficacy, we would expect *E*-β-ocimene to be efficiently learned at lower concentrations than β-myrcene. Likewise, since *racemic* linalool would activate at least two RN types (one with high and the other with low efficacy), we asked whether it might be learnt at lower concentrations than the two other odorants. Thus, the learning rate of the three odorants at different concentrations was compared. To clarify the relationship between RN activation and the strength of olfactory input to the brain, electroantennograms (EAG) were recorded as responses to the same concentrations of the three odorants. Finally, we asked whether the moths have a higher ability to discriminate odorants that activate different RN types than odorants activating the same type. Choosing concentrations that gave a similar learning rate in the previous experiment, differential conditioning experiments with all six odour pairs were carried out. The hypothesis was that moths would more easily discriminate *racemic* linalool from β-ocimene/β-myrcene than β-ocimene from β-myrcene, since the two latter odorants activate the same RN type.

Materials and methods

Insects and insect preparation

H. virescens (Fabricius 1777) (Heliothinae; Lepidoptera; Noctuidae) pupae originated from a laboratory culture at Syngenta, Basel, Switzerland. Females and males were placed in separate containers and kept in a phase-shifted light–dark photoperiod at 22°C. After emergence, the adult moths were placed in new containers and given sucrose solution *ad libitum*. Insects used in the experiments were 3–6 days old. For the conditions of the experiments, they were starved for 2 days before the experiments. All the animals were immobilised in plastic tubes (Experiment 1) or plexiglass holders (Experiment 2, 3 and 5) during the experiments, and could freely move their

proboscis and antennae. After being placed in the holders, all insects were tested for proboscis extension reflex by stimulating the antennae with sucrose solution (1 M). Only insects showing the reflex were included in the tests. The number of non-responding insects was usually between 10 and 30%. To allow the insects to adapt to the experimental environment, they were kept in the experimental room in dim light conditions for 1 h before the experiment started.

Test compounds

The odorants used as CS were the plant odorants β-ocimene (21% *Z*-β-ocimene and 58% *E*-β-ocimene), β-myrcene (75%), and *racemic* linalool (Experiment 1 and 2, 87% *racemic* linalool; or Experiment 3, 4 and 5, 82% *racemic* linalool). We chose to use the commercially available racemate of linalool, since both enantiomers activated the two RN types. The given purities of the odorants were determined by analyses of injections in a gas chromatograph with DB-wax column (J&W Scientific, Agilent Technologies, Palo Alto, CA, USA; 30 m, i.d. 0.25 mm, film thickness 0.25 μm). Separations in the column were performed from the initial temperature 80°C with an increase rate of 6°C min^{-1} to 180°C, and a further increase rate of 15°C min^{-1} to 220°C. Except for the *racemic* linalool (Sigma-Aldrich, Steinheim, Switzerland) used in Experiment 1 and 2, all odorants originated from Fluka Chemika (Sigma-Aldrich, Steinheim, Switzerland). For experiment 1, we applied 10, 100 and 1000 mg of undiluted *racemic* linalool onto pieces of filter paper inserted into glass cartridges. The lowest amount (1 mg) of *racemic* linalool was obtained from a dilution in n-hexane (>99%). In Experiments 2, 3, 4 and 5 the compounds were first diluted in hexane, and from each dilution 100 μl were evaporated onto a piece of filter paper placed in a weak stream of pure nitrogen (99.99%). Each filter paper was placed in a glass cartridge sealed with teflon caps. Using these cartridges, we stimulated the animals with the different concentrations of the three odorants. Sucrose (1 mol l^{-1}) was used as the US stimulus.

Experiment 1. Effect of CS concentration on acquisition and retention

As a first approach to conditioning experiments in moths we wanted to investigate the effect of CS concentration on acquisition and retention. For each conditioning trial the insects were placed one at a time into a purified air stream (~240 ml min^{-1}). The antennae were stimulated by blowing an air pulse (~50 ml min^{-1}, 5 s) through the test cartridge into the continuous air stream. After 2.5 s of the onset of the CS, the US was given for ~4 s. The US was always a compound US, and was given first to the antennae and then to the extended proboscis, which allowed an uptake of sucrose solution. If the moth did not show a response to the sucrose stimulation of the antenna during the first conditioning trial, extension of the proboscis was forced and was stimulated with the sucrose solution. The inter-trial interval (ITI) was set to 7 min, and 12 trials were performed. Insects that did not respond to the US in three subsequent training trials were considered to be not properly motivated, and were excluded from the analysis. The four concentrations of *racemic* linalool were tested in separate experiments for each sex. The results obtained for all concentrations were plotted as the percentage of animals showing the CR as a function of the number of conditioning trials (acquisition curves). Extinction tests were performed 15 and 120 min after the end of training.

Experiment 2. The effect of different inter-stimulus intervals (ISIs) on learning

One important parameter in learning experiments is the interval between CS and US. We therefore tested five groups for different ISIs to determine the optimal delay between onset of CS and US. The ISIs –1 (backward pairing), 0, 1, 2 and 3 s were chosen for this experiment according to previous results from the moth *S. littoralis* (Fan et al., 1997). *Racemic* linalool was used as the CS in a concentration of 100 mg on filter paper, which produced good acquisition in Experiment 1. Both males and females were used, but in separate test series. To adapt the insects to the test conditions, they were placed in the constant air stream (~400 ml min^{-1}) 15 s prior to the odour stimulation (~100 ml min^{-1}, 5 s) and left in this position until 5 s after the end of the US stimulation. The sucrose stimulus (US) was given for 5 s, first to the antennae and then to the extended proboscis. All ISIs were tested in all experiments. Each individual was given eight conditioning trials. Precise timing of the CS and US stimulation was ensured by an auditory signal given every second. The ITI was set to 15 min. Acquisition curves were made for the forward-paired groups (i.e. groups trained with the ISIs 1, 2 and 3 s). An extinction test was performed 60 min after the last conditioning trial. The results were plotted as the percentage of animals showing the CR in the extinction test for the different ISIs. Exclusion of non-motivated animals was carried out as in Experiment 1.

Experiment 3. Dose–response relationships of learning rate with racemic *linalool, β-ocimene and β-myrcene*

The effect of different concentrations of odorants used as CS on the learning rate was compared between the three odorants *racemic* linalool, β-myrcene and β-ocimene. Female moths were subjected to conditioning procedures with the three odorants at four different concentrations (0.1, 1.0, 10 and 100 mg of the component on filter paper). Ten conditioning trials with 1 s ISI and 15 min ITIs were performed. Extinction tests were performed 15 min after the last conditioning trial. Otherwise the experimental procedure was identical to that in Experiment 2. Since all four concentrations of linalool showed a high percentage of CR in the test, additional conditioning experiments were performed with a lower amount of this odorant (0.001 mg). The results obtained for the three odorants were plotted as the percentage of animals showing CR in the extinction test as a function of odorant concentrations.

Experiment 4. Electroantennogram dose–response relationships for racemic *linalool, β-ocimene and β-myrcene*

Since the three odorants caused different dose–response

relationships for the learning rates, EAG recordings were performed to find out whether these differences could be correlated with different antennal sensitivity to the odorants. Moths were immobilised with the antenna fastened at the base and the tip by tungsten hooks to a dental-wax layer on the top of the plexiglass holder. Glass capillary microelectrodes filled with Ringer's solution (150 mM NaCl, 3 mM $CaCl_2$, 3 mM KCl, 10 mM TES buffer, pH 6.9) were used as recording electrodes. The reference electrode was placed into the haemolymph of a proximal antennal segment or into the eye, and the recording electrode into the cut tip of the antennae. The three odorants *racemic* linalool, β-myrcene and β-ocimene were tested in five concentrations (from 0.01 to 100 mg) in two parallel series, starting with the lowest concentrations. Antennae of five females were tested, and in two of these a lower concentration of the three odorants (0.001 mg) was included. The glass cartridges used for a maximum of 2 days were stored at –20°C. A double set of control cartridges, each with hexane or pure air, were used as controls before each test series and between each concentration step. The preparation was placed in a constant clean air stream (~250 ml min^{-1}), which was turned off during stimulation. Stimulation was performed *via* a parallel air stream through the odour cartridges (~500 ml min^{-1}) onto the antenna. The time between each odour stimulus increased from 1 min for the lowest to 8 min for the highest concentrations to avoid adaptation. The signals were amplified 1000× and visualised and analysed using the EAG software (version 2.6d, Syntech, Hilversum, The Netherlands). The mean response to the pure air stimulus was subtracted from all odorant responses and the results were given as the percentage of the response to 10 mg *racemic* linalool, which elicited a high response in all animals. The results are given as dose–response curves of the average EAG response of the two parallel test series to each odorant concentration in all individuals tested. Finally, we plotted the learning rate for each concentration of each odorant (% proboscis extension in the extinction test in Experiment 3) as a function of the EAG response (% of the 10mg *racemic* linalool standard) and calculated the Pearson *R* correlation coefficient.

Experiment 5. Discrimination between racemic *linalool, β-ocimene and β-myrcene*

We investigated the ability of female moths to discriminate the three odorants, and evaluated whether there was a difference in discrimination between odorants activating the same RN type and odorants activating different RN types. Concentrations of the three odorants giving similar learning levels in Experiment 3 were chosen for this experiment: 0.1 mg for *racemic* linalool, 10 mg for β-ocimene and 100 mg for β-myrcene. Each animal was trained with two odorants, one rewarded (CS+) and one explicitly presented without a reward (CS–). Rewarded and unrewarded trials were performed six times each in a pseudo-randomised order (CS+, CS–, CS–, CS+, CS–, CS+, CS+, CS– or CS–, CS+, CS+, CS–, CS+, CS–, CS–, CS+). The ISI was set to 1 s and the ITI to 15 min. Fifteen minutes after the last conditioning trial, both odorants were presented in extinction tests in a balanced order.

Statistics

In all conditioning experiments, dichotomous data were recorded, the moths responding or not to the CS at each trial. We compared responses either within each group, to see the development of responses throughout the experimental procedures, or among groups, to compare moths' performance in different conditions.

Within-group comparisons

To see whether acquisition took place, i.e. if the responses within each group increased significantly throughout the conditioning procedure, we used Cochran's Q test (Experiments 1, 2 and 5). When testing for differences in the proportion of responding individuals within the same group, at two points in time or to two different stimuli, we used the exact McNemar test (Experiments 1 and 5).

Among-group comparisons

To compare the overall learning rate among groups, we counted the number of responses given by each moth during the whole conditioning procedure and used the Kruskal-Wallis test (Experiment 1). This test was also used in Experiment 3 to test for significant differences between concentrations of the three odorants giving the same degree of CR. When only two groups were involved, i.e. when comparing sexes in Experiment 1, the exact Mann-Whitney test was used. Moreover, to compare moths' responses in the test phase, a Fisher's exact test (*Nd.f.)* was used on the proportion of responding insects in the different groups (Experiments 1, 2, 3 and 5). In Experiment 2, we also carried out pair-wise comparisons between groups conditioned with different ISIs, using Fisher's exact tests with Bonferroni correction. With an overall significance level of 5% and 10 combinations of groups being tested, the new threshold for each test was set to 0.5% (5%/10).

To compare EAG responses to the three odorants at the five highest odorant concentrations (Experiment 4) a General Linear Model with repeated measurements test was used (sphericity assumed) with a Tukey (honestly significant difference) correction (threshold 5%), SPSS software (versions 11.0 and 12.0) was used for all the statistical analyses. The correlation between the learning rate (% proboscis extension in the extinction test in Experiment 3) and the EAG response (% of the 10 mg *racemic* linalool standard) for each odorant concentration was estimated by the Pearson *R* linear correlation coefficient.

Results

Experiment 1. Effect of CS concentration on acquisition and retention

In the first conditioning experiments, 162 females and 237 males were tested and both sexes showed an increase in PER to the odorant, after repeated paired presentations of odorant and sucrose reward. No spontaneous responses to the odorant were

observed at the first conditioning trial. In both sexes, responses significantly increased during conditioning trials for all four concentrations of *racemic* linalool (Fig. 1A,B; Cochran's Q test, $P<0.05$, 11 d.f.), except for the group of males trained to the lowest concentration of 1 mg (Cochran's Q test; $P>0.05$; 11 d.f.). When comparing the overall response levels to the different concentrations, significant heterogeneity was found for both sexes (Kruskal-Wallis test, $P<0.05$, 3 d.f.). Comparison of the response levels between the two sexes within each concentration, showed no significant differences (exact Mann Whitney test, $P>0.05$).

Extinction trials were performed for all individuals 15 and 120 min after the last conditioning trial (Fig. 1C,D). The group conditioned to the highest odorant concentration (1000 mg) showed the highest response level after 15 min (60% of the females and 53% of the males). A significant decrease of the response level with decreased odorant concentration at the 15 min test was found for both sexes (Fisher's exact test, $P<0.05$, 3 d.f.). However after 120 min a tendency for differences was seen only among the male groups; no significant differences were observed among the female groups (Fisher's exact test, $P=0.055$ and $P>0.05$, 3 d.f., respectively). By comparing the response level between the sexes at the 15 min and the 120 min tests, no significant differences were found at any concentration (Fisher's exact test, $P>0.05$, 3 d.f.). A general decrease of the response level was found during the period of 15 to 120 min after the last conditioning trial. However, the decrease was only significant for one trained concentration in the females (1000 mg) and another one in the males (10 mg) (exact McNemar test, $P<0.05$).

Experiment 2. The effect of different inter-stimulus intervals (ISI) on learning

We found in Experiment 1 that PER increased with repeated presentations of CS and US paired with an ISI of 2.5 s. We wanted to check the associative nature of this conditioning, and find the optimal ISI for building the CS–US association. In each sex, five groups of moths were subjected to eight conditioning trials with different ISIs (–1, 0, 1, 2 and 3 s). Owing to the different durations of CS–alone presentation in each trial, it was not possible to compare acquisition curves. For 1 and 2 s ISIs, responses increased significantly during training (data not shown, Cochran's Q tests, $P<0.05$ in both sexes). For an ISI of 3 s there were no significant performance increases in females, and only a trend in males ($P>0.05$ and $P=0.06$, respectively). Acquisition could not be recorded in the groups with –1, 0 and 1 s ISI, because the odorant never appeared before the US.

Responses of the different groups in the extinction test could be compared directly because all groups received the same odorant stimulation (Fig. 2A,B). In both sexes, a clear heterogeneity appeared among the different ISI groups

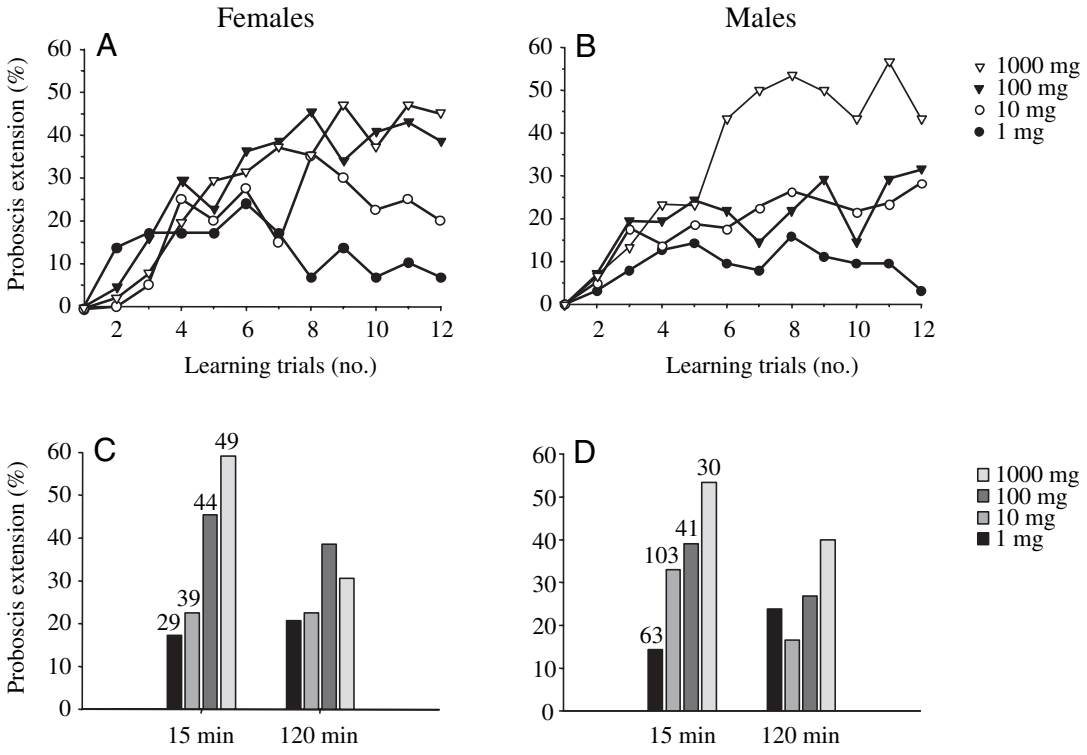

Fig. 1. Acquisition curves obtained in classical conditioning experiment with four concentrations of *racemic* linalool associated with a sucrose reward in *H. virescens* females (A) and males (B). The proportion (%) of insects showing the PER in each of the 12 conditioning trials is shown. The ISI was 2.5 s. (C,D) The proportion of insects with PER at different points of time (15 and 120 min) after the conditioning trials. The number of individuals included in each group is given above the bars for the 15 min test. An increased percentage of both females and males showed the PER when trained to increasing *racemic* linalool concentrations.

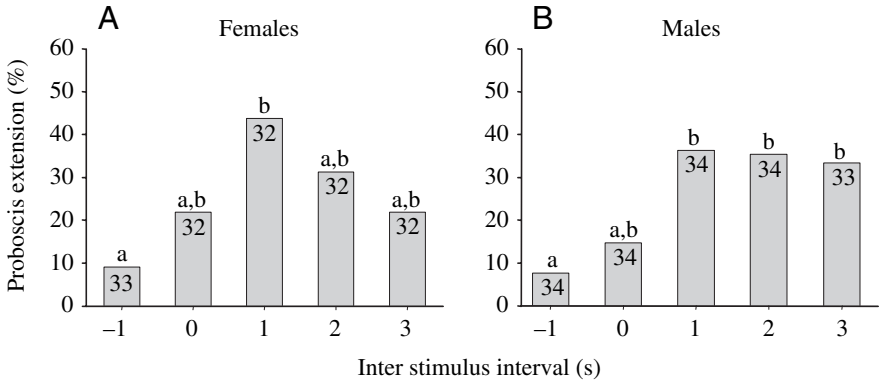

Fig. 2. The proportion of *H. virescens* females (A) and males (B) that showed the PER 1 h after conditioning experiments using 100 mg *racemic* linalool associated with a sucrose reward at five different ISIs (–1, 0, 1, 2 and 3 s). The interval of 1 s gave the best performance with 44% of the females and 36% of the males responding to the odorant. The groups differing significantly from each other (Fisher's exact test with Bonferroni correction; $P<0.005$) are marked with 'a' *vs* 'b'. The number of insects in each group is given within the bars.

(Fisher's exact test, $P<0.05$, 4 d.f.). The presentation of the US one second before the CS (backward pairing) resulted in the lowest percentage of responses, whereas the CS presented one second before the onset of the US gave the highest number of responses. Longer intervals of 2 and 3 s showed a decreased percentage of responding females, whereas in males no change was seen in responding individuals for these intervals. In both sexes, pair-wise comparisons between ISI groups showed a clear significant difference between the group of 1 s ISI and the group –1 s ISI (Fisher's exact test with Bonferroni correction, $P<0.005$ in both sexes). This difference between responses in the forward- and in the backward-pairing groups was a critical control for showing that PER performance in our experiments was due to the formation of a CS–US association, i.e. to associative learning. Based on these results, the ISI of 1 s was chosen for Experiments 3 and 5. No significant differences were found between the results obtained for each ISI in the two sexes (Fisher's exact test, $P>0.05$).

Experiment 3. Dose–response relationships of learning rate with racemic *linalool, β-ocimene and β-myrcene*

As in Experiment 1, where we found that the learning rate increased with increasing concentrations of *racemic* linalool, we compared in this experiment dose–response relationships for the three odorants, *racemic* linalool, β-ocimene and β-myrcene. In these experiments, no spontaneous responses to any of the odorants were ever observed. Moreover, due to the fact that moths are slow to extend the proboscis, and that the ISI was 1 s, very few moths showed CR during the conditioning procedure. Thus, only the results of the extinction tests (5 s odour presentations, allowing the moths to respond) performed 15 min after the conditioning trials, were used and are shown in Fig. 3A. The results of the extinction tests generally showed an increase of responses to the CS with increasing concentrations of the three odorants. This dose–response effect was significant for β-myrcene (Fisher's exact test, $P<0.05$), but not for the two other odorants (Fisher's exact test, $P>0.05$). However, the concentration-dependent PER curves differed between the odorants. A much lower concentration threshold (~0.001 mg) was found for *racemic* linalool than for β-ocimene (~0.1 mg) and β-myrcene (~1.0 mg). The CR to *racemic* linalool reached maximum already at the concentration of 0.1 mg. Higher concentrations of β-ocimene and β-myrcene were required for learning; β-ocimene showing qualitatively higher percentages of CR than β-myrcene at the three concentrations of 0.1, 1.0 and 10.0 mg.

Experiment 4. Electroantennogram dose–response relationships for racemic *linalool, β-ocimene and β-myrcene*

Since we found dose–response relationships for the three odorants in the conditioning experiments, we evaluated antennal sensitivity to the same stimuli using EAG recordings. Fig. 3B shows the dose–response curves obtained for the three odorants *racemic* linalool, β-ocimene and β-myrcene. The results showed that the moths were more sensitive to *racemic* linalool than to the two other odorants, with the strongest response increase from 0.1 mg to 1 mg. The other two odorants elicited increased responses from the concentration of 1 mg, reaching maximum responses at 10 mg. At the five highest concentrations (0.01 mg to 100 mg) a stronger response was obtained to *racemic* linalool than to the two other odorants. The differences were significant between the responses to *racemic* linalool and β-myrcene [General Linear Model with repeated measurements test and a Tukey (honestly significant difference) correction, ($P<0.05$)] and showed a trend toward significant difference ($P=0.07$) between the responses to *racemic* linalool and β-ocimene. No significant difference was found between the β-ocimene and β-myrcene responses ($P>0.05$). The average response to evaporated hexane on filter paper is also indicated in Fig. 3B, being slightly higher than the responses to the air controls. The same trend toward sensitivity was found for the EAG recordings and conditioning of the moths, being more sensitive to *racemic* linalool, than to β-ocimene, and, finally, least sensitive to β-myrcene. When representing conditioning performance relative to EAG responses (Fig. 3C), a clear and significant correlation (the Pearson R correlation coefficient, $R^2=0.46$, $P<0.05$) was found.

Experiment 5. Discrimination between racemic *linalool, β-ocimene and β-myrcene*

We compared how the three odorants were differentiated from each other using differential conditioning experiments in which one odorant was rewarded (CS+) and a second one was

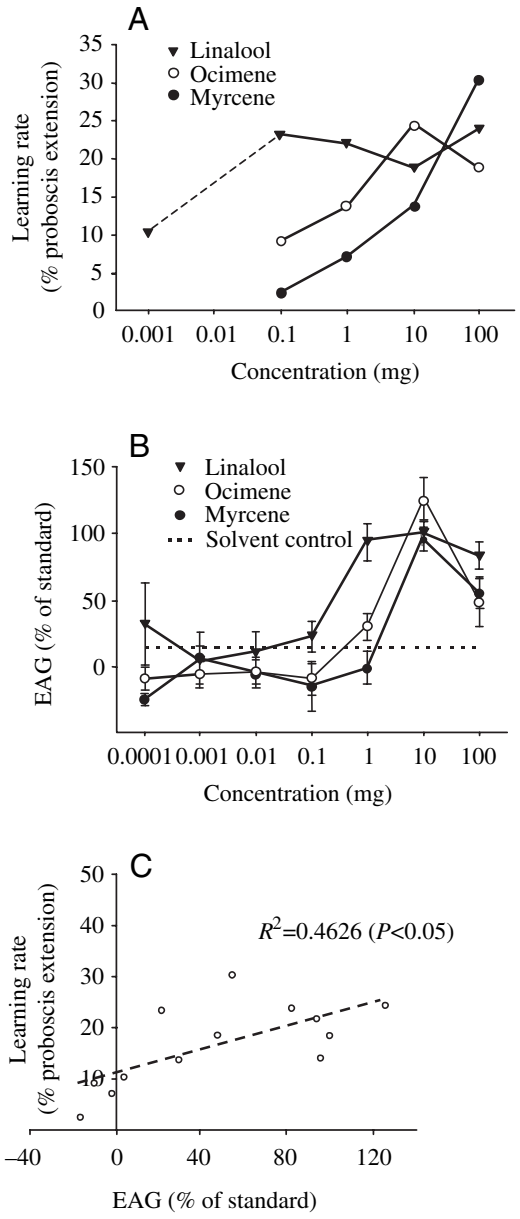

Fig. 3. (A) Learning rate of *H. virescens* females showing the conditioned response (proboscis extension) to different concentrations of the odorants *racemic* linalool, β-ocimene and β-myrcene in extinction tests. The tests were performed 15 min after a 10-conditioning trial procedure. A lower threshold for learning and for the maximum proportion of insects responding were obtained for *racemic* linalool as compared to the two other odorants. Insect numbers were 36–39 for all groups except for the additional group conditioned to *racemic* linalool at 0.001 mg (N=49). (B) Dose–response relations as electroantennograms (EAG) for the different concentrations of the three odorants *racemic* linalool, β-ocimene and β-myrcene obtained in *H. virescens* females. The responses are given as percentages of the response to 10 mg *racemic* linalool and as the average of the responses to the two parallel test series in five female moth antennae. The lowest concentration was tested in two individuals only. The mean response to the control (air) was subtracted from the odorant responses, which explains the slightly negative response values for some of the lower odorant concentrations. The dotted line shows the mean response to the other control (hexane evaporated on filter paper). Error bars indicate the standard error of means. (C) The learning rate (% proboscis extension in A) plotted as a function of the EAG response (% of the 10mg *racemic* linalool standard in B) showed a significant correlation (Pearson R correlation coefficient).

explicitly non-rewarded (CS–). To be able to compare differentiation between odorants controlling for different odour saliencies, we chose three concentrations of the odorants inducing similar learning levels in Experiment 3: moths trained with concentrations of 0.1 mg of *racemic* linalool, 10 mg of β-ocimene and 100 mg of β-myrcene, showed the same level of CR (26%, 27% and 34%, respectively, exact Kruskal-Wallis test, P>0.05, 2 d.f.). Thus, these concentrations of the three compounds were chosen for this experiment. During the differential conditioning procedure the moths showed only minimal responses to the presentations of the CS+ or of the CS– (data not shown, Cochran's Q test in all cases non-significant). This low response level to the CS+ was probably due to the short ISI (1 s, as in Experiment 3) in addition to the higher complexity of this differential conditioning procedure for moths. The results of the extinction tests performed 15 min after differential conditioning are shown in Fig. 4. Pooling the responses to CS+ and CS– in each of the three odorant pairs, irrespective of which odorant was the CS+, showed that the moths responded significantly more to the CS+ than to the CS– (Fig. 4A, exact McNemar test, P<0.05). When separating the responses within each odorant pair, we could evaluate possible asymmetries in response differentiation when each odorant was a CS+ or a CS– (Fig. 4B). When comparing CS+ and CS– responses in the six different odorant combinations, we only found significant differences in three cases: $CS+_{myrcene}$/$CS-_{linalool}$, $CS+_{myrcene}$/$CS-_{ocimene}$, $CS+_{ocimene}$/$CS-_{myrcene}$ (exact McNemar test, P<0.05). Comparing responses with the CS+ in the two combinations of each odour pair, we found a significant difference for the linalool/myrcene pair. These findings point to an asymmetry between linalool and myrcene, although we corrected for potential concentration effects.

Discussion

The present study has shown important characteristics of olfactory conditioning in the moth *H. virescens*, using three plant odorants. Two of these odorants, β-myrcene and β-ocimene activate the same RN type and the third odorant, *racemic* linalool, activates two different RN types. Using all three odorants, the maximum percentage of moths (~50%) found to learn the CS–US association was relatively low as compared to the proportion usually found in the honeybee (80–90%, Bitterman et al., 1983). In addition, a higher number of conditioning trials appears to be needed in the moth to be able to reach an asymptote in CRs. Whereas three conditioning trials are usually sufficient in the honeybee, moths needed at least eight trials, as shown in the acquisition curves of Experiment 1

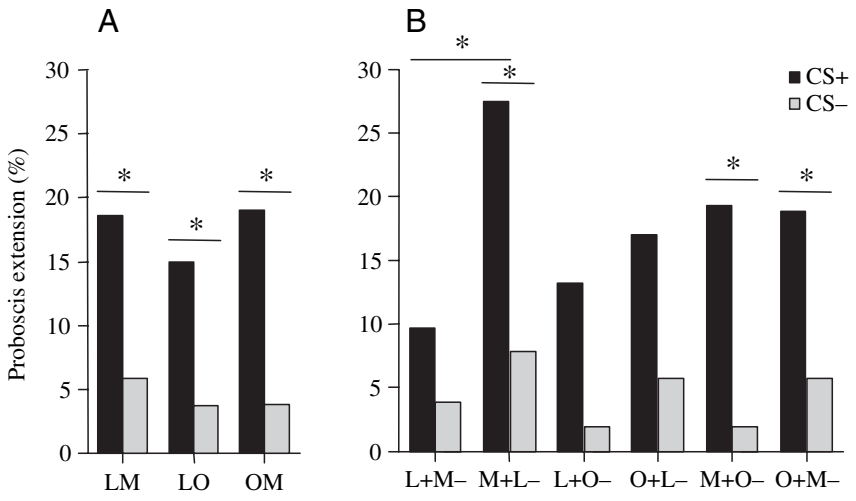

Fig. 4. (A) Learning rate of *H. virescens* females showing the conditioned response (proboscis extension) to CS+ and CS– (irrespective of which odorant was the CS+) in extinction tests after differential conditioning with the three odorant pairs [*racemic* linalool (L) and β-myrcene (M), L and β-ocimene (O) and O and M, 12 conditioning trials, N=103–106 for each pair)]. The concentrations used were 0.1 mg *racemic* linalool, 10 mg β-ocimene and 100 mg β-myrcene. The moths responded significantly more to the CS+ in all cases (exact McNemar test, P<0.05). (B) Learning rate in extinction tests after differential conditioning with the six pairs of three odorants [each odorant (CS+ and CS–) was presented pseudo-randomised six times, N=51-53 for each pair]. There was a significant discrimination between the CS+ and CS– in the M+L–, M+O– and O+M– groups (exact McNemar test, P>0.05, marked with an asterisk). For the odour pair M L, an asymmetry appeared as the insects showed a significantly higher discrimination when M was the rewarded odorant than when L was the rewarded odorant CS+ (Fisher's exact test, P<0.05).

(Fig. 1). Also, in a preliminary study of this moth species, several conditioning trials (five) were necessary and the percentage of moths showing CR was relatively low (Hartlieb, 1996). In contrast to the results of this previous moth study, which showed lower learning performance in males than in females, the present study found no indication for a difference in learning ability between the sexes. In the present study we attempted to define optimal experimental conditions to ameliorate conditioning rates. One critical parameter in learning experiments is the interval between CS and US onsets. The ISI effect usually shows a typical bell-shaped curve; too long or too short intervals do not allow the formation of a CS–US association, or, in the case of backward training, inducing an inhibitory association (Hellstern et al., 1998). We explicitly addressed this question in Experiment 2 and found that ISIs of 1–3 s gave the best performance (Fig. 2). Other parameters that could explain relatively low conditioning rates would be the overall motivation of the animals. By discarding moths that repeatedly did not respond to the US during conditioning, we ensured that the moths kept in the analysis were appetitively motivated and drank the sucrose solution given at the proboscis. However, we cannot be sure that the starvation period of 2 days produces the highest possible motivation. We will continue to look for ameliorations to our experimental conditions to reach higher response levels. However, we have to keep in mind that lower learning ability in this species, as compared to other insect models, could also be an inherent characteristic of moths. In comparison with the social honeybees, for instance, with their longer life span and the need for efficient foraging for their over-wintering colonies, it may not be surprising that this species has evolved better learning abilities than the short-living solitary moths.

In Experiments 3 and 4, we used an ISI of 1 s between CS and US, as it gave the best learning performance. However, it appeared to be an important drawback, because it did not allow us to record moths' acquisition performance. During conditioning, we usually measure learning performance at each trial, when presenting the odour CS alone, before the US. In moths, which have a long proboscis and tend to respond slowly, CS–induced responses could usually not be recorded within 1 s, producing very low acquisition curves. In contrast, moths conditioned with 1 s ISIs showed response levels as high as 35–45% in the test trials, in which the odorant was presented for 5 s (Fig. 2A,B). We thus believe that such short ISIs are rather impracticable with moths, and future experiments should use a somewhat longer ISI (e.g. ISI of 2 to 3 s), which also support good learning performance.

Another important parameter in olfactory learning is its dependency on CS concentration. The present study showed that increased odorant concentrations resulted in a higher percentage of moths showing the CR (Figs 1A,B, 3A). Higher odour concentrations have also been shown to increase the percentage of moths showing the CR (increased cibarial pump response) in *M. sexta* (Daly et al., 2001) and to increase discrimination and induce better memory consolidation in PER conditioning in *A. mellifera* (Bhagavan and Smith, 1997; Pelz et al., 1997; Wright and Smith, 2004). In the present study, moths showed the ability to learn *racemic* linalool at a much lower concentration than the other two odorants. Already at the concentration of 0.001 mg, conditioning to *racemic* linalool took place and maximum proportions of CR were reached after training with concentrations of 0.1 mg, as shown by Experiment 3 (Fig. 3A). In comparison, the highest proportion of CR to β-ocimene and β-myrcene was reached at the 10 mg and 100 mg concentrations, respectively. These concentration differences in learning performance may be partly due to a higher sensitivity of the moth olfactory system to linalool than to the other two odorants, as was indicated by the significant correlation of learning rate with the amplitude of EAGs recorded in Experiment 4 (Fig. 3). This finding is also in accordance with the increased responses to higher odorant concentrations obtained from single RNs measured by electrophysiological recordings (Stranden et al.,

2003b; T. Røstelien, M.S., A.-K. Borg-Karlson and H.M., unpublished) and with increased calcium responses obtained in the antennal lobe in optical imaging recordings (Skiri et al., 2004; Stranden et al., 2003b). Since single-cell recordings have shown similar sensitivities of the RN types to their primary odorants (Stranden et al., 2003b), the present EAG recordings may indicate that *H. virescens* has a higher number of RNs responding to linalool than to the other two odorants. In addition, the EAG and calcium imaging experiments showed somewhat stronger responses to β-ocimene than to β-myrcene, the two compounds known from electrophysiological recordings to activate the same RN type, β-ocimene being the primary and β-myrcene a secondary odorant (Røstelien et al., 2000b; Stranden et al., 2003b). Thus, the results of the present study may indicate that learning performance with the three odorants increases with a larger number of RNs responding and/or with a higher firing rate of the RNs.

Electrophysiological studies have previously shown that β-ocimene and β-myrcene activate the same RN type, whereas linalool activates two other types; linalool being the primary odorant of one and a secondary odorant of the other (Røstelien et al., 2000b; Stranden et al., 2003b; T. Røstelien, M.S., A.-K. Borg-Karlson and H.M., unpublished). One would therefore expect the moths to discriminate between linalool and the two other compounds more easily than between β-ocimene and β-myrcene. Our conditioning experiments indicated that the moths do indeed have the ability to discriminate between all three odorants (although not as well in all cases), including between β-ocimene and β-myrcene (Fig. 4). This is surprising, since the concentrations of β-ocimene and β-myrcene used should induce the same spiking rate in the RNs (i.e. we used 10x more β-myrcene than β-ocimene). It is likely that discrimination between these two odorants is due to the activation of other RN types by only one of the compounds, although this has not yet been found in electrophysiological recordings. In fact, in a calcium imaging study, one or two glomeruli in males were activated by β-ocimene but not by β-myrcene (Skiri et al., 2004). Considering the number of ordinary glomeruli (61–63) in the antennal lobe of the heliothine species that are believed to receive plant odour information (Berg et al., 2002; H.T.S., B. G. Berg and H.M., unpublished) and the 19 RN types identified so far (T. Røstelien, M.S., A.-K. Borg-Karlson and H.M., unpublished), we think that not all RN types have yet been described. Future work will attempt to broaden our knowledge of RN types on *H. virescens* antennae. Alternatively, since we used commercially available chemicals, we cannot exclude that impurities in the high concentrations tested may have contributed to the discrimination between the two test stimuli β-ocimene and β-myrcene.

A significant discrimination asymmetry was found in Experiment 5. Moths learned to discriminate the pair $CS+_{myrcene}/CS-_{linalool}$ but not the pair $CS+_{linalool}/CS-_{myrcene}$, whereas they learned to discriminate equally well the pair β-myrcene/β-ocimene, irrespective of which odorant was trained (Fig. 4B). Since the odorant concentrations showing equal acquisition were chosen, the asymmetry in the β-myrcene/*racemic* linalool discrimination tests cannot be explained on the basis of different sensitivities to these odorants. Sensory similarity (or difference) should be indicated by the amount of differential responding, and should be independent of which element of an odour pair is being used as the CS+ or CS–. Discrimination values depend on the stimulus trained, and here stimulus strength can be excluded as a parameter. The better-learned stimulus differs qualitatively in its potential to gain the properties of a learned odour. This potential is called the 'salience' of a stimulus in learning theory (Rescorla and Wagner, 1972). Salience differences become apparent when the pair-wise comparison between CS+ and CS– responses of the two odorants indicates an asymmetry, assigning a higher salience to the odorant that is responded to with higher probability when it is used as a CS+ than as a CS–. It is not surprising that a rank order of salience is not simply additive, as found in Experiment 5 (β-myrcene=β-ocimene, β-myrcene>*racemic* linalool but not β-ocimene>*racemic* linalool). This means that salience is not an isolated parameter of a stimulus, but depends on the conditions under which the stimulus is learned, here the not rewarded odorant in a differential conditioning paradigm.

In conclusion, the present study has shown that performance in PER conditioning in moths depends on the concentration of the odorant CS as well as on the precise timing between CS and US presentations, and has identified conditions in which PER conditioning is relatively efficient. Also, we have shown that three different odorants, inducing quite different dose–responses in EAG experiments (this study) and in calcium imaging experiments (Skiri et al., 2004) also had different thresholds in the learning experiments, in such a way that the EAG dose–response relationship could predict the learning rate. By using a differential conditioning procedure, we found that moths could discriminate all three odorants, which was surprising given the fact that two odorants, β-ocimene and β-myrcene, activate the same RNs. Furthermore, we found that differential conditioning of odorant pairs leads to discrimination values that are biased by differences in the salience of the stimuli, even if the stimuli are made subjectively equally strong. The present results thus show that moths can be used to answer specific questions about how olfactory learning performance in insects relates to odorant detection, processing and perception. The development of coupled electrophysiological recordings and behavioural experiments on moths would be critical in this endeavour.

Abbreviations

CR	conditioned response
CS	conditioned stimulus
EAG	electroantennogram
ISI	inter-stimulus interval
ITI	inter-trial interval
PER	proboscis extension response
RN	receptor neurone
US	unconditioned stimulus

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