

ASSOCIATIVE OLFACTORY LEARNING IN THE MOTH *MANDUCA SEXTA*

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Summary

The proboscis extension response conditioning protocol has been used to explore olfactory-based associative learning in an array of insects. We have monitored a different feeding reflex, which involves activation of the cibarial pump, to demonstrate olfactory learning in the moth *Manduca sexta*. In the first experiment, four different treatment conditions were used to assess associative (Pavlovian) learning. The results indicate that an excitatory cibarial pump response develops and is retained for at least 24 h only when odor is forward-paired with the presentation of sucrose. Three control treatments, backward pairing, air (no odor) pairing and random pairing, failed to increase the cibarial pump response. However, an excitatory cibarial pump response developed when the backward- and air-paired groups received

forward pairing of odor and sucrose on the following day. In contrast, moths experiencing random pairing on day 1 displayed a slower rate of acquisition during forward pairing on day 2, which may indicate inhibition. The second experiment investigated discrimination learning. Two odors were randomly presented, one odor being forward-paired with sucrose (+), the other presented alone (–) in a counterbalanced design. Again, only when odor was forward-paired with sucrose did learning occur. We discuss the implication of these findings for a broader comparative analysis of learning in insects.

Key words: cibarial pump response, moth, *Manduca sexta*, learning, olfaction, feeding reflex, conditioning.

Introduction

Animals live in a complex and dynamic matrix of stimulus events. While innate behavioral responses to some of these events are imperative to survival, in many cases there is also a distinct advantage for these responses to exhibit some degree of plasticity based on prior experience. In the noctuid moth *Manduca sexta*, olfactory stimuli are used to navigate successfully to nectar-bearing flowers, to host plants for oviposition and to conspecifics for mating (Christensen and Hildebrand, 1987a,b). Adult male moths emerge fully able to navigate along the wind-borne pheromone plumes produced by females. In contrast, the highly diverse and dynamic range of olfactory cues that signal the presence of host plants for oviposition and for nectar require that moths modify their innate tendencies to compensate for changing environmental conditions. To optimize foraging, adult moths, like honeybees (von Frisch, 1967), must learn which olfactory cues from myriad blends of plant volatiles signify the presence of a valued resource.

There is a substantial body of evidence demonstrating that insects can modify innate behavioral responses by forming associations between relatively novel cues and biologically relevant environmental events. For instance, parasitoid wasps are able to form associations between volatile odorants from the dung of hosts and other host-specific non-volatile waste products (Lewis et al., 1991). They also form associations

between host-plant volatiles and host location/oviposition (Kaas et al., 1990; Kerguelen and Cardé, 1996; Zanen and Cardé, 1991). Tully and Quinn (1985) have successfully demonstrated conditioned aversion by associating odor with electric shock in *Drosophila melanogaster*. Raubenheimer and Tucker (1997) found that *Locusta migratoria* can learn that color and odor cues signal the presence of either protein- or carbohydrate-rich foods.

Much of the research in insect learning has centered on conditioning the proboscis extension response (PER; Menzel, 1990). This reflexive response can be elicited in many nectar-consuming insects, such as the honeybee (Kuwabara, 1957). Simply touching the antennae with sucrose solution elicits extension of the mouthparts and activation of feeding behavior. In PER conditioning, a novel conditioned stimulus (CS) is paired with sucrose presentation (unconditioned stimulus or US). Proboscis extension response conditioning is a simple yet powerful Pavlovian protocol that provides an ideal experimental platform for in-depth parametric analysis of conditioning in insects. It has been used to explore olfactory learning in the honeybee *Apis mellifera* with respect to an array of learning phenomena including stimulus intensity (Bhagavan and Smith, 1997), blocking (Smith and Cobey, 1994; Couvillon et al., 1997; Smith, 1997), generalization and discrimination (Smith and Menzel, 1989). PER-based

conditioning has also been used to explore conditioning in an array of insect species including *Drosophila melanogaster* (Lofdahl et al., 1992). Recent investigations clearly demonstrate olfactory learning in three species of moth, *Helicoverpa armigera*, *Heliothis virescens* (Hartlieb, 1996) and *Spodoptera littoralis* (Fan et al., 1997).

In recent years, an agricultural pest moth, *Manduca sexta*, has been the focus of intense behavioral and neurophysiological analysis in relation to olfaction and olfactory processing (Christensen and Hildebrand, 1987a,b, 1988; Nighorn et al., 1998). This work has led to an understanding of the olfactory processing mechanisms underlying upwind orientation and flight response to pheromones and to floral and host-plant olfactory cues. This makes *M. sexta* an ideal candidate for studying the neurological foundations of learning. Indeed, the similarity between antennal lobe structure and olfactory processing of this moth and that of the vertebrate olfactory bulb (Shepherd, 1991; Shipley and Ennis, 1996; Hildebrand and Shepherd, 1997; Hildebrand et al., 1997), as well as the structural and functional similarity of its antennal lobe to that of other insects, provides a great opportunity for comparative analysis of olfactory learning and memory and the underlying neurophysiological processes that control these types of behavior.

Our goal was therefore to develop a protocol in which to evaluate the changes in moth behavior that arise from differential stimulation of the olfactory system with respect to food reinforcement. The first objective was to establish that *M. sexta* could learn to respond appetitively to novel olfactory cues as a result of associative processes. One method that can be used to assess the relative contributions of associative and non-associative processes to the modulation of the feeding behavior of moths in response to olfactory cues is to systematically manipulate CS-US contiguity (the temporal relationship between CS and US) and CS-US contingency (the predictive value that CS has upon US presentation). Excitatory associative processes require that the change in response be specific to forward pairing of CS and US (that is the presentation of the CS just prior to the presentation of the US). Other pairing conditions such as backward pairing of CS and US, where contingency is reversed, will not produce an increase in response strength to the CS unless non-associative processes such as sensitization are also co-modulating behavior. Furthermore, by degrading the contingency and temporal contiguity of the CS-US relationship through the random pairing of CS and US, little or no predictive value will be provided by the CS and, hence, excitatory associations will not form. In this randomly paired condition, there is a possibility of excitatory associations forming because random patterns can offer some predictability. However, with repeated trials, this effect becomes increasingly small. A decrease in response strength produced by random pairing, over and above that seen in backward pairing, could also indicate inhibitory processes.

A second approach that can be used in assessing associative

processes is to demonstrate discrimination learning. Discrimination learning bolsters the associative learning argument by demonstrating that subjects can differentially increase responsiveness selectively to odors on the basis of the predictive value of each odor in relation to the US. Selective responsiveness to meaningfully different stimuli is the foundation of discrimination learning and provides strong evidence that observed changes in behavior are not due entirely to sensitization. Furthermore, discrimination analysis provides a framework within which a detailed understanding of sensory processing can be achieved.

Materials and methods

Subjects

Male and female *Manduca sexta* were obtained during middle- and late-stage pupal development from Arizona Research Labs, Division of Neurobiology (ARLDN). Rearing conditions have been described in detail elsewhere (Bell and Joachim, 1976). Pupae were classified by sex, placed individually in brown paper bags and stored until used in environmental chambers at 28 °C, 70 % relative humidity and on a 16 h:8 h L:D cycle.

Pupae were checked once daily, just before initiation of the dark cycle, and the date was recorded on the bags in which newly emerged pupae were found. Adult subjects were held without food or water for a minimum of 5 days after the eclosion date before they were used in any of the treatment conditions described below. Three procedural points must be addressed here. First, subjects were monitored according to age to ensure complete development of all sensory systems. Second, we observed that holding subjects without food for several days increased their motivation to feed and increased the level of conditioned responsiveness in the experimental paradigm. Third, each subject was placed in only one of the treatment conditions described below and was used for only one series of training trials.

Preparation

Subjects were placed into individual plastic preparation tubes measuring 7 cm in length and 1.5 cm in diameter. A portion of the tubing on the top had been cut away, leaving a 1 cm high by 0.5 cm wide tab used to assist in restraining the subject. On the back of the tube, a four-wire female-ended plug with two electrode leads was permanently attached. Multiple insect preparations could then be plugged into a magazine for conditioning.

The subject was placed in the tube from the bottom and forced to the top. Once the head and part of the thorax had emerged from the top of the tube, a length of pipe cleaner was wrapped around the thorax, and the subject was twist-tied to the plastic tab extending from the tube. This procedure effectively restricted the subject without harming it. An additional piece of masking tape was placed just under the head and over the exposed portion of the thorax, further restricting the subject and preventing the legs from disturbing the

electrode preparations. The proboscis was then extended and threaded through a 4 cm length (0.05 in inner diameter) of Tygon flexible surgical tubing, leaving the distal portion of the proboscis exposed. This tubing was then affixed to the front of the preparation tube with a soft wax. Mounting the proboscis in this manner made it easier to deliver the sucrose solution with greater temporal accuracy.

A Teflon-coated fine silver wire electrode was placed just under the surface of the head capsule, between the compound eye and the sagittal mid-line, bringing it into contact with the cibarial pump muscle. A reference electrode was placed into the contralateral compound eye. The preparation tube was plugged into a multi-port magazine used to train up to five animals in sequence.

The magazine was built from a 10 cm × 55 cm length of Plexiglas. Attached to the Plexiglas sheet were five 1 cm tall Plexiglas dowels around which the plastic preparation tubes could be mounted. Also attached to the Plexiglas sheet was the male end of the plug where leads from each preparation tube made contact. Leads from the magazine connected to an A-M Systems (model 1700) differential AC amplifier. Output from this amplifier led to an oscilloscope, to an audio output device and to an A/D board in a computer.

The conditioning stage consisted of an odor cartridge made from a 1 ml tuberculin glass syringe. Either geraniol (3 μl) or 1-hexanol (3 μl) was placed on a piece of filter paper and inserted into the syringe. These two floral compounds were selected because of their prior successful use in studies of conditioning in honeybees (Thorn and Smith, 1997; Smith and Cobey, 1994; Bhagavan and Smith, 1997; Smith, 1998) rather than any potential biological relevance to *M. sexta*. In addition, we chose arbitrarily high concentrations of each odor solely on the basis of the work on bees and irrespective of what might be regarded as 'biologically relevant concentrations' for *M. sexta*. The odorant cartridge was then placed in the front of the stage and connected to an aquarium pump air supply. A computer-controlled shunt allowed airflow either to an exhaust or through the odor cartridge. The cartridge was then aimed at an exhaust port located approximately 10 cm away.

During conditioning trials, subjects were placed in the center of the stage so that the odor cartridge was aimed directly at the head approximately 9 cm away. The exhaust produced a gentle air stream in which the subject was centered. When the shunt was opened, odorant was blown gently into the air stream over the head and antennae of the subject and then into the exhaust vent. An auditory signal indicated the initiation and termination of the presentation of sucrose, which was applied to the proboscis by hand. During test trials, odor was controlled in the same manner, but sucrose solution was not presented.

Response measurements

The cibarial pump reflex was elicited by application of the unconditioned stimulus (US; sucrose solution) directly onto the partially extended proboscis. Responses were measured as changes in the rate of electromyographic (EMG) activity recorded from the cibarial pump muscle, a procedure similar

to that described by Smith and Menzel (1989) in the honeybee. Subjects were scored according to detected changes in feeding behavior in response to presentation of the conditioned stimulus (CS). This score was based on three indicators: output from the audio output device attached to the amplifier, visualization of the EMG recording during a trial on the oscilloscope, and extension of the tip of the proboscis that protruded from the end of the plastic tube. Specifically, if any of these indicators showed an increase in activity as a result of presentation of the CS, a response was recorded for that trial. These observational data are used below only as an index of acquisition of the learned response. However, this measure is necessarily confounded by the US presentation 3 s after CS onset and therefore underestimates the conditioned response. In addition, we do not expect a correlation between the acquisition data and the test or extinction trials (below) because the increase in latency of the conditioned response increases as a function of repeated conditioning trials. Acquisition data were only collected during trials that were forward-paired or during test trials that involved presentation of odor without reinforcement. Other types of pairing, which varied the temporal relationship between the CS and US, inflate estimates of the conditioned response (CR) by confounding it with the unconditioned response (UR).

During test trials, EMG activity of the cibarial pump muscle was digitized at 2.5 kHz and stored on disk. Recordings were made at three points during conditioning on both day 1 and day 2 (see below): once prior to conditioning, once midway through conditioning and once immediately following conditioning. Each recording was 10 s in duration, so that EMG activity was sampled for approximately 3 s before odor presentation, for the 4 s of odor presentation and for approximately 3 s after the termination of odor presentation. Pre- and post-presentation recording times varied slightly as a function of computer performance.

Spikes were counted for each EMG recording using a spike-counting application created in HP-VEE. A 30 μV threshold was used to differentiate activity of the cibarial pump muscle from noise and other activity (see Fig. 2). Detection of a spike was based on a positive then negative threshold crossing within a set time. The number of spikes was counted over two time periods: one prior to the onset of the CS, and one from the onset of the CS until the end of the trial. Spike counts were converted to pre-CS and post-CS frequencies by dividing the number of spikes by the respective time period.

A small increase in the pre-CS frequency was detected across trials. This increase could have been due to a number of factors, including sensitization to CS/US and/or residual sucrose in and around the proboscis. We could not conclusively demonstrate the underlying causes of this change in baseline responsiveness from the post-CS data, so we chose to extract it by subtracting the pre-CS frequency from the post-CS frequency. By subtracting the pre-CS activity, a net change in cibarial pump reflex activity in response to the CS is produced. All statistical analyses were performed on the basis of this change in frequency from pre- to post-CS presentation.

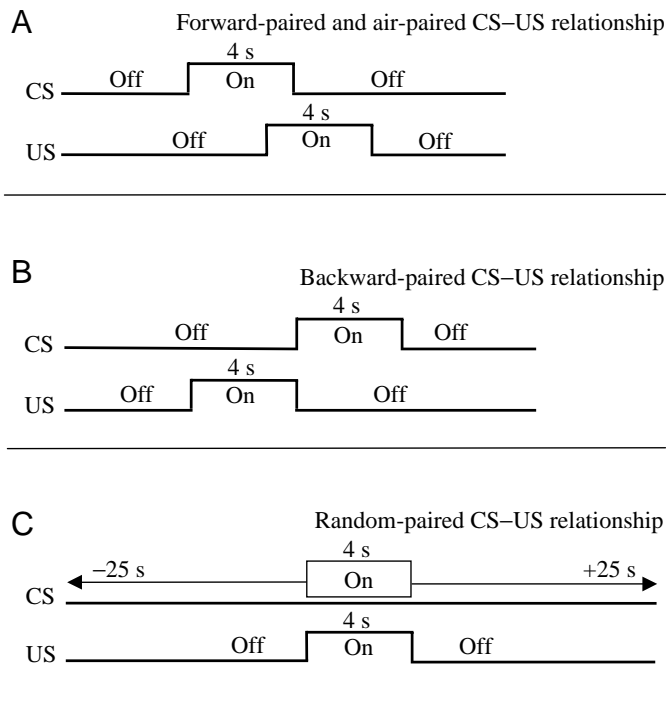


Fig. 1. Basic conditioned stimulus/unconditioned stimulus (CS-US) pairing conditions used to assess olfactory learning. (A) The forward-paired (FP) and air-paired (AP) treatments first receive a stimulus from the odor or blank cartridge (conditioned stimulus, CS; 4 s) and are then presented with sucrose (unconditioned stimulus, US; 4 s). Note the 1 s overlap between CS and US. (B) The backward-paired (BP) condition received the US first (4 s) followed by the CS (4 s) with no overlap. (C) The random-paired (RP) condition received the CS at a random interval (± 25 s) with respect to the US.

Experiment 1: analysis of associative and non-associative effects

In total, 160 subjects were conditioned using a traditional Pavlovian-style conditioning paradigm. The procedure described below was adapted from the proboscis extension response (PER) conditioning protocol in the honeybee (Menzel and Bitterman, 1983). Groups of 20 subjects were assigned to one of four groups and conditioned with one of the two odorants. On the first day, all groups received the following series of three test (T) and ten conditioning (C) trials, T1-C1-C2-C3-C4-C5-T2-C6-C7-C8-C9-C10-T3. The test (extinction trials) measured the baseline responsiveness to the CS (T1) and subsequent acquisition after either five (T2) or 10 (T3) conditioning trials. During test trials, the CS was presented alone, and EMG activity was recorded to disk. Conditioning trials were treatment-specific (see below).

Fig. 1 illustrates the four treatment-specific conditioning schemes used in this experiment. The forward-paired (FP) treatment group (Fig. 1A) received a 4 s pulse of air from the odorant cartridge (CS) directed at the antennae of the subject. At 3 s into CS presentation, $1 \mu\text{l}$ of a 1.25 mol l^{-1} sucrose solution (US) was touched to the exposed tip of the proboscis

for 4 s. Most of the subjects were able to consume the entire droplet during that period. Note that there was a 1 s overlap between the CS and US.

The air-paired (AP) treatment (Fig. 1A) investigates the context-specific learning effects of mechanosensory and otherwise unspecified stimuli that accompany odorant and sucrose delivery. Individuals in the AP treatment condition received the identical forward-pairing conditioning described above. However, no odorant was placed in the cartridge. Hence, the AP group experienced only the airflow that normally occurs with the onset of the CS presentation and experimenter movement that occurs prior to and during US presentation.

In the backward-paired (BP) treatment group (Fig. 1B), subjects received 4 s of sucrose delivery ($1 \mu\text{l}$ maximum) prior to 4 s of odor presentation. There was no explicit overlap of CS and US presentations in the backward-paired treatment. However, it is likely that residual sucrose was still present on the proboscis after sucrose presentation terminated, creating some stimulus overlap.

The fourth treatment group received random-paired (RP) presentations of sucrose and odorant (Fig. 1C). In this treatment group, presentations of the CS were randomly spaced, between +25 and -25 s, around presentations of the US. Thus, on average, half the conditioning trials were forward-paired and half were backward-paired. Note that, in the forward-paired and air-paired groups, US delivery is contingent on prior CS delivery and that in the backward-paired group the contingency is reversed. However, temporal contiguity is maintained in the forward-paired, air-paired and backward-paired groups. In the random-paired group, there is neither consistent positive contingency nor temporal contiguity between CS and US. The advantage of this approach is that the same number of CS and US stimuli are given over the same total training period, making direct comparison between the random-paired treatment condition and other conditions more meaningful. Note too that this procedure differs from explicit unpairing (Hartlieb, 1996; Fan et al., 1997) in which the CS and US are randomly presented in separate trials. One disadvantage with our method is that, because it is roughly equivalent to the truly random procedure (Rescorla, 1969), there were trials in which close CS-US contiguity and positive CS-US contingency occur, which could produce some excitatory learning.

On the second day, all groups of subjects received the same combination of test and conditioning trials as on day 1; however, all conditioning trials were forward-paired. On day 1, the purpose was to differentiate the efficacy of the various treatment conditions. Day 2 was used to demonstrate that differences between groups on day 1 were due to the different treatment conditions and not to chance inter-individual differences (recognizing, however, that treatment received on day 1 affects the efficacy of the treatment given on day 2).

Experiment 2: discrimination learning analysis

The goal of this experiment was to demonstrate discrimination learning. Specifically, when subjects are

reinforced with sucrose to one odor (A+) they should increase responsiveness to that odor while simultaneously not increasing responsiveness to a second odor (B-) that they experienced as often as the A+ odor but without reinforcement. Twenty subjects received pseudo-randomized trials exposing them to both 1-hexanol and geraniol for an equal number of times. Ten of the subjects received 1-hexanol followed by sucrose (A+) in six forward-paired trials exactly as described in experiment 1. Pseudo-randomly interspersed with the A+ trials, the subjects received six additional trials during which they were exposed to geraniol alone (B-). The second group of 10 subjects received the same procedure; however, in this group, geraniol was paired with sucrose (A+) and 1-hexanol was presented alone (B-). The patterns of pseudo-random presentation used were as follows: AB-ABBABAABABBA-AB-AB and BA-ABBABAABABBA-BA-BA. Note that the pre- and post-tests, separated from conditioning trials by dashes, were reversed as a counterbalancing measure.

As in experiment 1, a 6 min inter-trial interval was maintained. Observational data (recorded as 0 or 1, see below) were collected for all trials in the same manner as described for experiment 1. Cibarial pump muscle activity in response to extinction trials with both the A+ and B- odors was also recorded at three times: 6 min prior to conditioning, 6 min after completion of conditioning, and 24 h after conditioning. The initial measurements recorded prior to conditioning provide the baseline responsiveness to each odor, while the post-conditioning measurements, taken both immediately and 24 h post-conditioning, provide a measurement of both discrimination learning and its retention. In addition, on the second day, 12 unconditioned extinction trials (no sucrose reinforcement), six for each odor, were given to all subjects. In these trials, A and B odors were presented in the same pseudo-randomized order. Again, 6 min inter-trial intervals were maintained and 0/1 observational data were recorded for each trial.

Statistical analyses

Electromyographic data were analyzed using the general linear model (GLM) and regression (REG) procedures in SAS (SAS Institute, 1996). The GLM procedure was used because it hierarchically partitions variance components, which allowed us to pre-specify causal priority while eliminating the need for *post-hoc* data analysis (Cohen and Cohen, 1983). Furthermore, in GLM analysis, the theoretical assumptions about transformations of variables can be hierarchically tested. The regression procedure was used to provide slope and intercept information. Data were subdivided by day and analyzed separately. Where some data were missing, individuals were excluded from the analysis, leaving each group with at least 18 complete records.

A number of additional variables were collected for the analysis. SEX, was coded 0 for a male or 1 for a female and was treated as a continuous variable. Treating this main effect as a continuous variable enabled us to investigate the effect of sex *via* differences in the *y*-intercept. AGE represents age in

days (post-eclosion) at the initiation of treatment. AGE ranged from 5 to 9 days, with a mean of 7.25 days. Most subjects, however, were between 6 and 7 days old at the start of the experiment. ODOR was a continuous variable (coded 0 or 1) differentiating 1-hexanol from geraniol. Each subject was given a unique identification number, which formed a class variable accounting for individual differences (INDIVIDUAL DIFF). The logarithmic function of the number of trials was used in the analyses because typically in conditioning experiments the initial learning is fast and then tapers to a maximum. Thus, a logarithmic function more accurately models acquisition curves than a linear function.

From these variables, a number of transformations and interactions were produced to test specific hypotheses. TREATMENT(1) was a rank-ordered variable that differentiated the four conditioning groups for day 1. Forward-paired groups were coded 2, random-paired groups 1, backward-paired groups 1 and air-paired groups 0. This rank ordering reflects two points. First, the forward-paired treatment displayed an elevated initial response relative to the other groups in spite of the fact that all groups were treated identically at this point. Here, by explicitly coding for this difference in initial responsiveness, we extract variance that might covary with our measurement of acquisition and thereby remove this as a possible confounding effect. The second reason for creating the TREATMENT(1) ordinal scale effect was because the air-paired group did not receive a highly salient olfactory cue, and their baseline responsiveness should therefore be weak. A significant effect would support the above theoretical assumptions.

On day 2, different expectations arise. TREATMENT(2) is a second rank-ordered variable that reflects the hypothesis that the air-paired and backward-paired groups will express the same intercepts if the absence of response in these groups reflects an absence of learning on day 1. Furthermore, subjects in the random-paired group should have learned that the presentation of the CS does not predict the coming presentation of sucrose. Thus, the TREATMENT(2) variable was coded as follows: forward-paired, 2; backward-paired, 1; air-paired, 1; and random-paired, 0. Again, a significant TREATMENT(2) effect would support the above theoretical assumptions.

Because learning curves typically display a ceiling effect across trials, the effect of trials was log-transformed (LOG TRIAL) to model an asymptotic function for acquisition. To explore whether the forward-paired group showed an increase in response level relative to the air-paired, backward-paired and random-paired groups, the CONTRAST variable was created. Here, the forward-paired treatment was juxtaposed to the backward-paired, random-paired and air-paired groups. As a main effect, CONTRAST is collinear with the ordinal variable TREATMENT(1) and therefore was not used as such. However, the interaction between CONTRAST and LOG TRIAL specifically tests the hypothesis that the slope of the forward-paired group across trials is different from those of the other three groups.

Additional transformations were created for the second

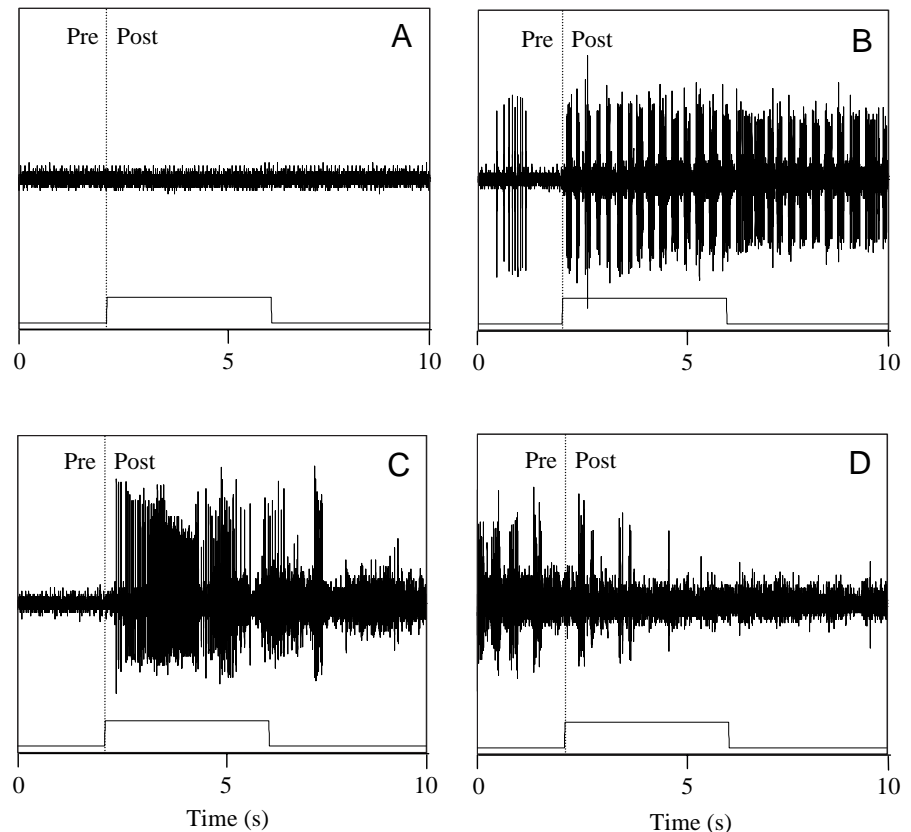


Fig. 2. Electromyographic (EMG) recordings displaying examples of conditioned responses (A) prior to training for all groups, (B) for forward-paired (FP) mid-training on day 1 and for forward-paired, backward-paired (BP) and air-paired (AP) mid-training on day 2, (C) for forward-paired 24 h post-training, and (D) for backward-paired and Random-paired (RP) conditions mid-training on day 1. Pre and post delineate activity prior to the onset of the conditioned stimulus (CS) and after CS onset. The step in the lower trace indicates that the CS is on for 4 s.

experiment. First, a contrast-coded variable, BASELINE VS B⁻, was created to test the hypothesis that the response of the subjects to the B⁻ odor does not change as a function of repeated trials. This variable was contrasted as follows: all initial recordings, irrespective of odor received, were coded as -1; all B⁻ post-test measurements, irrespective of the odorant, were coded as +1; all other trials were disregarded. POST VS B⁻, BASELINE is a contrast-coded variable that juxtaposes the A⁺ conditions to all other trials and is coded as follows: A⁺ is coded as +1; baseline is coded as -1; B⁻ is coded as -1. The theoretical assumption here is that there is no difference between baseline and the B⁻ condition, an assumption that was tested empirically (see above).

Results

Fig. 2 displays four 10 s examples of EMG recordings that demonstrate a number of points. The first recording (Fig. 2A) is typical of activity prior to conditioning, when there is little or no change in activity in response to the CS odorant. There were occasional exceptions in which subjects responded spontaneously to the CS, but the magnitude of this response was typically small relative to the response produced by the US. The second recording (Fig. 2B) illustrates a typical response to the CS after forward-paired conditioning. Note that there is a very short latency between the onset of the CS and a dramatic increase in EMG activity lasting for many seconds. After the termination of the recording session, this activity frequently continued in increasingly shorter bursts that could

carry on for a few minutes. In this figure, there was also some EMG activity prior to CS onset. As discussed above, these random bursts of activity may be due to residual sucrose from prior trials or possibly to the accrual of sensitization. Fig. 2C is an example of a learned response to the initial CS presentation on day 2. Note that, unlike Fig. 2B, there is no pre-CS activity and subjects have not received sucrose or odorant for 24 h. Nevertheless, upon presentation of the odorant, a strong conditioned response occurs.

Fig. 2D is typical of the responses recorded in the backward-paired treatment group upon presentation of the CS after training. Note that, in this recording, the onset of the CS demarcated a decrease in activity. This pattern of decreasing activity was more frequent in the backward-paired group, but also occurred in the random-paired group. The important point to note is that, while some increase in spontaneous activity might have been detected prior to CS presentation, CS presentation frequently had either no effect or a negative effect on response strength in these groups.

Day 1: an increase in response to odorant is specific to forward pairing

The percentage of subjects that increased their feeding activity in response to geraniol (Fig. 3A) and 1-hexanol (Fig. 3C) presentation for day 1 was calculated from the observational data and is displayed by treatment condition. No data were displayed for the backward-paired treatment group because acquisition data could only be collected during the test trials. Fig. 3A,C shows a distinct increase in response to the

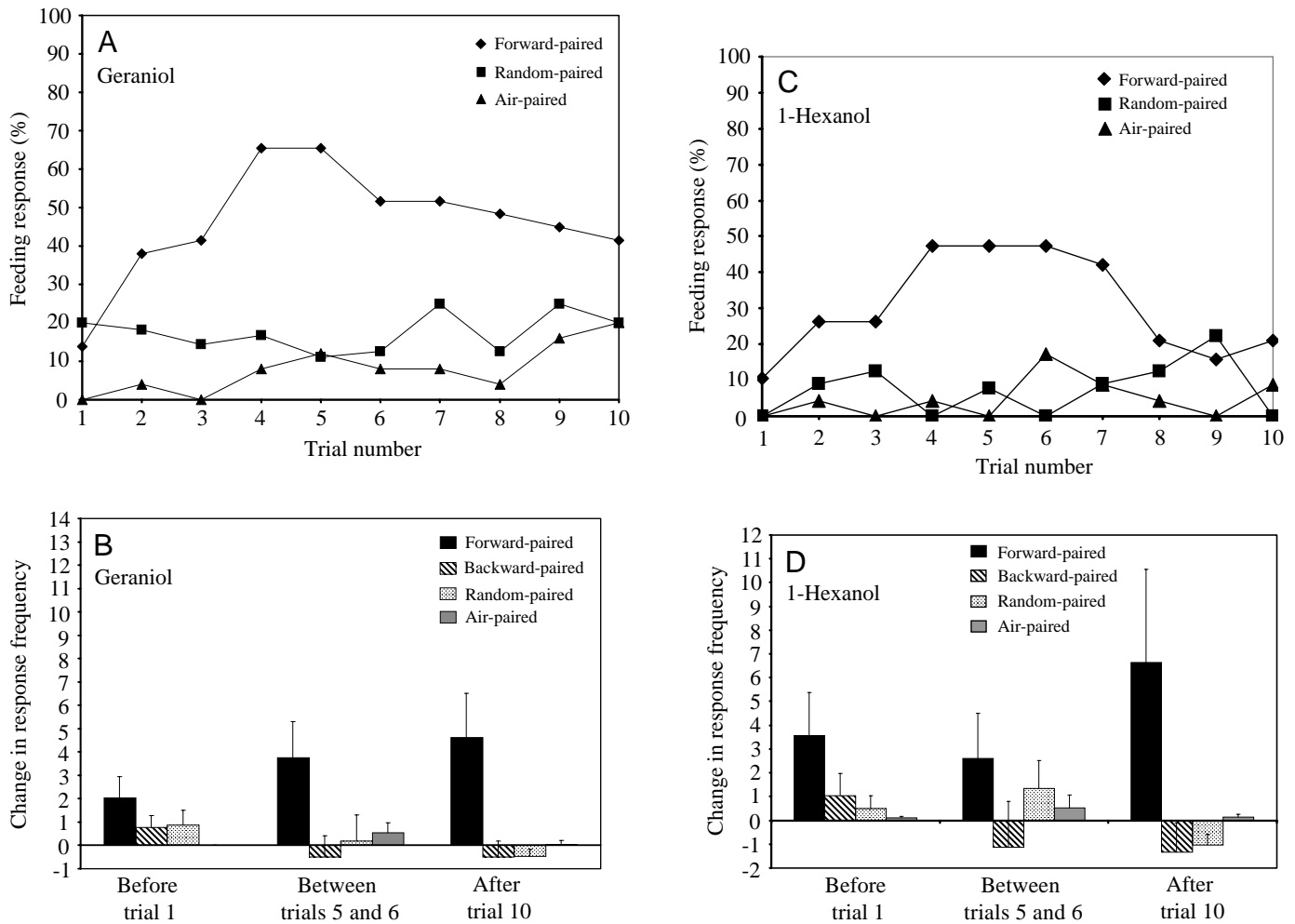


Fig. 3. Measurements of the conditioned cibarial pump muscle reflex response to odor presentation on day 1. (A,C) Acquisition for the air-paired (AP) ($N=23$), forward-paired (FP) ($N=18$) and random-paired (RP) ($N=19$) (backward-paired, BP; $N=18$, not applicable) conditions as a percentage of subjects responding to geraniol (A) or 1-hexanol (C) after the onset of the conditioned stimulus (CS) but prior to presentation of the unconditioned stimulus (US). (B,D) Mean change in spike activity from the cibarial pump muscle in response to odor presentation prior to trial 1, after trial 5 and after trial 10 for geraniol (B) and 1-hexanol (D). Note that the initial mean change in spike activity for the air-paired group was zero (B). Values are means + S.E.M.

odorant in the forward-paired condition up to trial 6, after which the response drops off. This appears to contradict the EMG data, which show a general increase in CR response strength (Fig. 3B,D). This apparent discrepancy may be due to the increasing latency of the CR over trials. On reinforced trials, as the CR latency increased beyond 3 s after CS onset, it became confounded with the US presentation and the ensuing UR; thus, we were unable to score a positive CR. On test trials, the US was not presented, so additional time was given during which the CR could be registered. These two response measurements are therefore not completely comparable.

Fig. 3A,C shows that the air-paired and random-paired conditions show very little change as trial number increases. Furthermore, the generally weaker response in the air-paired group on day 1 signifies the importance of odor in this learning procedure. Fig. 3B,D also shows the mean change in spike frequency in response to CS presentation during the test trials for geraniol (Fig. 3B) and 1-hexanol (Fig. 3D). The response

strength to odor presentation increased in the forward-paired treatment across trials, while the other treatment conditions tend to vary around a zero response.

Statistical analysis of the EMG data collected from the cibarial pump muscle support the above observations. Table 1 displays the results from GLM models for day 1. The overall model for day 1 produced a significant F -value of 3.14 ($P<0.005$) and explained 62.1% of the total variation in the response of the subjects to the CS. The main effects of AGE, SEX and ODOR were not significant. The absence of statistical interactions between SEX, AGE and ODOR with LOG TRIAL indicates that acquisition rate does not change as a function of these variables. However, TREATMENT(1) was significant, explaining 4.6% of the model variance. The regression equation at the bottom of Table 1 reveals a positive increase in intercept, indicating that the forward-paired condition had the highest intercept, followed by those for the backward-paired and random-paired groups. As expected, INDIVIDUAL

Table 1. Results of general linear modeling and analyses for experiment 1, day 1

Variable	NDF, DDF	Type I SS	R SQ	F value	Probability
Model	161	8792.79	0.623	3.14	0.0001**
Error	309	5447.28			
SEX	1, 152	26.76	0	0.51	NS
AGE	1, 152	12.89	0	0.25	NS
ODOR	1, 152	1.38	0	0.03	NS
TREATMENT(1)	1, 152	661.77	0.046	12.87	0.0005**
INDIVIDUAL DIFF	152, 309	7910.83	0.555	2.95	0.0001**
LOG TRIAL	1, 309	0	0	0	NS
SEX × LOG TRIAL	1, 309	19.08	0	1.08	NS
AGE × LOG TRIAL	1, 309	2.46	0	0.14	NS
ODOR × LOG TRIAL	1, 309	4.95	0	0.28	NS
TREATMENT(1) × LOG TRIAL	1, 309	241.45	0.017	13.87	0.0002**

Regression:

$$\text{Delta frequency} = 0.71 + 0.27\text{TREATMENT}(1) + 1.18(\text{CONTRAST} \times \text{LOG TRIAL})$$

SEX is coded 0 for male and 1 for female.

AGE is a continuous variable representing age at the start of training, ranging from 5 to 9 days post-eclosion.

ODOR is coded 0 for 1-hexanol and 1 for geraniol.

TREATMENT(1) is a rank-ordered scale that is formatted as follows: forward-paired, 2; backward-paired, 1; random-paired, 1; air-paired, 0.

INDIVIDUAL DIFF is a categorical variable representing individual differences.

LOG TRIAL is the log-transformed function of trial.

CONTRAST is contrast-coded variable that juxtaposes the forward-paired group with all control groups.

The regression analysis displays the intercept and the standardized value of β for all significant continuous variables.

NS, not significant. ** $P \leq 0.005$.

NDF, numerator degrees of freedom; DDF, denominator degrees of freedom; SS, sum of squares; R SQ, proportion of total variance explained. Delta frequency, change in response frequency.

DIFF was also significant and accounted for 55.5 % of the explained variance, indicating that a substantial amount of the variability in cibarial pump reflex activity is attributable to individual differences. The TREATMENT(1) × LOG TRIAL interaction was also significant, explaining 1.7 % of the total variance. The strong positive slope of the regression indicates that the forward-paired group showed a much greater increase in response strength to the CS across trials compared with the other groups. This relative difference indicates that associative processes contribute strongly to behavior.

Day 2: air-paired, backward-paired and random-paired groups increase response strength with forward pairing of CS and US

Fig. 4A,C displays the percentage of subjects exhibiting increased feeding behavior in response to geraniol and 1-hexanol presentation by group for day 2. Note that, while groups are referred to as air-paired, backward-paired, etc., on day 2, all groups received the forward-paired treatment to confirm that it was the differential treatment on the previous day that had produced observed differences in behavior and not an effect of individuals within the group *per se*. For both odors on the second day, the forward-paired group started at a point near the peak response level for day 1 and well above the final response level for day 1. This group continued to maintain a relatively constant level of response throughout the remaining trials. Both the air-paired and the backward-paired groups

showed a rapid increase in response strength to CS presentation across trials. In addition, the air-paired group displayed a stronger initial response to the CS than on day 1, and the subjects in this group progressively increased their responsiveness when exposed to more forward pairings of odorant and sucrose. This increase in responsiveness reflects the addition of a salient olfactory cue. In contrast, the random-paired group displayed a retarded response to CS presentation that persisted across trials. This pattern can also be seen in EMG recordings during test trials (Fig. 4C,D). The high initial response strength, observed in the forward-paired group, reveals robust 24h retention of learned response. The air-paired and backward-paired groups showed a low initial response that then increased to levels comparable with that of the forward-paired group on day 1. The random-paired group, however, showed substantially lower response levels.

Table 2 displays the GLM and regression analysis for day 2 data. This model was significant, with an F -value of 2.26 ($P < 0.0001$), and explained 54.1 % of the total variance in moth feeding behavior on the second day. The model on day 2 is somewhat different from that used on day 1. First, the model incorporated the hypothesis that subjects in the random-paired condition display a retarded response level to the CS, indicative of inhibitory learning. For this reason, we created the TREATMENT(2) variable as opposed to the TREATMENT(1) variable used in the analysis of day 1. TREATMENT(2) was found to be significant, accounting for 2 % of the total model

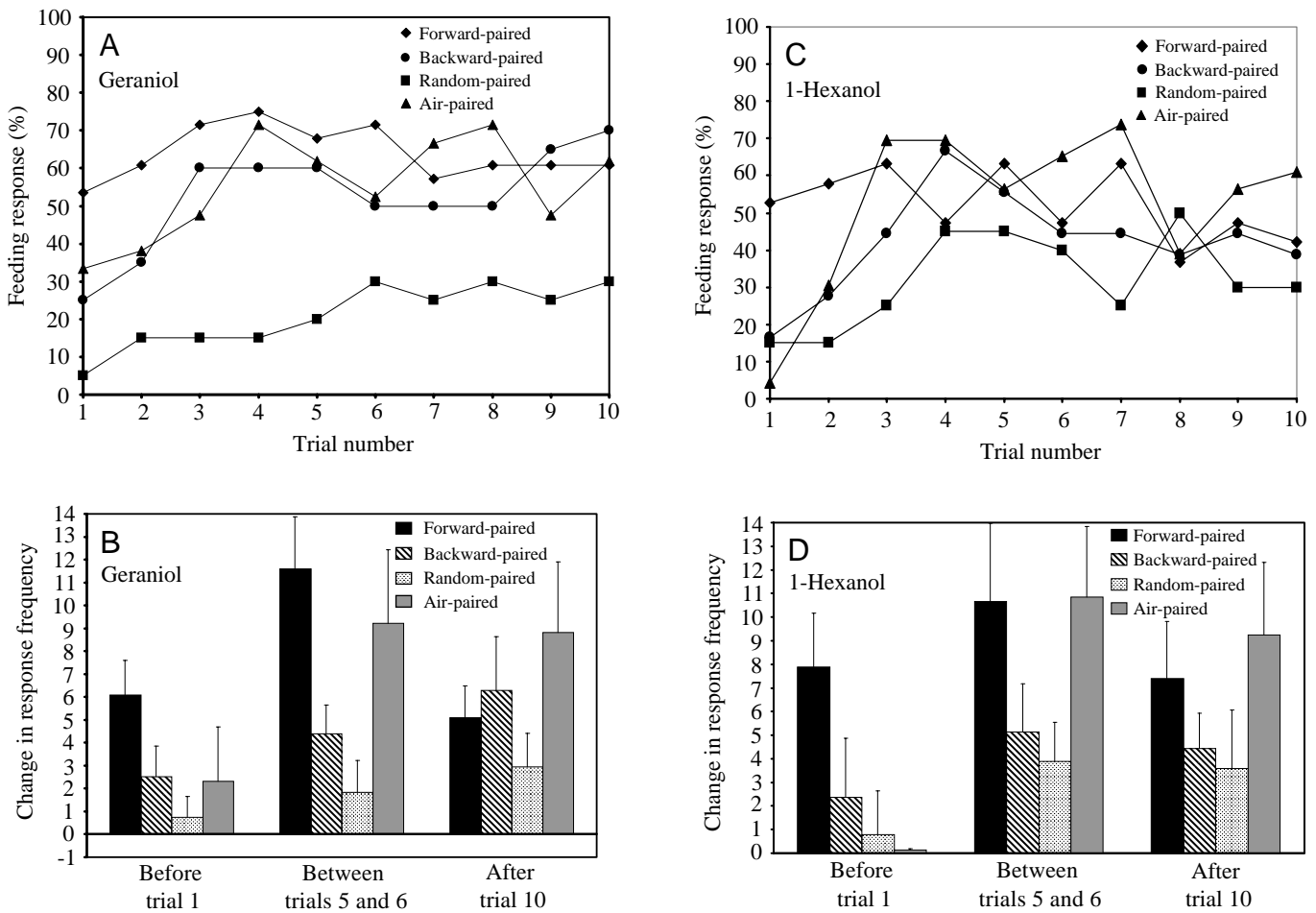


Fig. 4. Measurements of the conditioned cibarial pump muscle reflex response to odor presentation on day 2. (A,C) Acquisition for the air-paired (AP) ($N=23$), backward-paired (BP) ($N=18$), forward-paired (FP) ($N=18$) and random-paired (RP) ($N=19$) conditions as a percentage of subjects responding to geraniol (A) and 1-hexanol (B) after the onset of the conditioned stimulus (CS) but prior to presentation of the unconditioned stimulus (US). (B,D) Mean change in spike activity from the cibarial pump muscle in response to odor presentation prior to trial 1, after trial 5 and after trial 10 for geraniol (B) and 1-hexanol (D). Values are means + S.E.M.

variance. Regression analysis indicates that the forward-paired group has the highest y-intercept, followed by the backward-paired and air-paired groups and then by the random-paired group. Second, the expectation on day 2 is that all subjects in all groups should exhibit an increase in responsiveness to the CS. While LOG TRIAL was not significant on day 1, it was on day 2 ($P<0.005$), explaining 1.3% of the variance. This reflects the fact that response strength did not increase across trials for the backward-paired, random-paired and air-paired groups on day 1, but it did increase on day 2. The regression analysis indicates a strong positive effect of LOG TRIAL on day 2.

INDIVIDUAL DIFF was again significant, explaining 48.5% of the total variance and further indicating the importance of individual differences in feeding behavior. Finally, there was a significant interaction between TREATMENT(2) and LOG TRIAL. This interaction is an index of differences in slope between the forward-paired group and the other groups. Regression analysis indicates that, on day 2, there is a reversal of relative slopes. Whereas on day 1 the

forward-paired treatment group displayed strong learning, as indicated by the sharp increase in response strength, regression analysis on day 2 indicates a steeper positive slope in the air-paired, backward-paired and random-paired groups which are now receiving the forward-paired regime. This reversal occurred because the forward-paired treatment group had already attained a maximum response level on day 1. This suggestion is further supported by the higher intercept for the forward-paired group on day 2.

Moths can discriminate between odors

Fig. 5 displays the percentage of subjects that responded to the A+ and B- conditions for geraniol and 1-hexanol, respectively, on day 1 (acquisition phase) and on day 2 (retention/extinction phase) for the second experiment. For both odorants, the A+ condition yields an increase in response that is retained through to the following day, while the B- condition produces no such increase. This pattern is indicative of discrimination learning.

Fig. 6A,B shows changes in spike frequency before and after training for days 1 and 2. Fig. 6A displays the data with

Table 2. Results of general linear model and analyses for experiment 1, day 2

Variable	NDF, DDF	Type I SS	R SQ	F value	Probability
Model	161	30501.51	0.541	2.26	0.0001**
Error	309	26341.28			
SEX	1, 152	254.26	0	1.40	NS
AGE	1, 152	611.56	0.010	3.37	0.0683
ODOR	1, 152	29.99	0	0.17	NS
TREATMENT(2)	1, 152	998.33	0.018	5.50	0.0203*
INDIVIDUAL DIFF	152, 309	27579.61	0.485	2.13	0.0001**
LOG TRIAL	1, 309	786.53	0.013	9.23	0.0026**
SEX × LOG TRIAL	1, 309	3.37	0	0.04	NS
AGE × LOG TRIAL	1, 309	114.58	0	1.34	NS
ODOR × LOG TRIAL	1, 309	25.48	0	0.30	NS
TREATMENT(2) × LOG TRIAL	1, 309	327.56	0.006	3.88	0.0499*

Regression:

$$\text{Delta frequency} = -11.02 + 3.71\text{TREATMENT}(2) + 7.77\text{LOG TRIAL} + -0.44(\text{CONTRAST} \times \text{LOG TRIAL})$$

SEX is coded 0 for male and 1 for female.

AGE is a continuous variable representing age at the start of training, ranging from 5 to 9 days post-eclosion.

ODOR is a coded 0 for 1-hexanol and 1 for geraniol.

TREATMENT(2) is a rank-ordered scale that is formatted as follows: forward-paired, 2; backward-paired, 1; random-paired, 1; air-paired, 0.

INDIVIDUAL DIFF is a categorical variable representing individual differences.

LOG TRIAL is the log-transformed function of trial.

CONTRAST is contrast-coded variable that juxtaposes the forward-paired group with all control groups.

The regression analysis displays the intercept and the standardized value of β for all significant continuous variables.

NS, not significant. * $P \leq 0.05$; ** $P \leq 0.005$.

For further details, see Table 1.

1-hexanol in the A+ condition. There is a strong increase in response strength to 1-hexanol after training that is largely retained the following day, while responses to geraniol (B-) remain unchanged. Fig. 6B shows the same pattern with subjects demonstrating an increased response to geraniol (A+) after training that is retained on day 2, while the response to 1-hexanol becomes weaker. Note that 1-hexanol has a net negative spike frequency on day 1.

Table 3 displays the results of a GLM investigating discrimination learning for all subjects. The model is significant, with an F -value of 4.36 ($P < 0.0001$), and explains 49.7% of the total variance. Table 3 shows that there is no difference in response strength to the B- odor above baseline, indicating that the response strength of the subjects to the B- odorant did not change. The POST VS B- BASELINE variable is clearly significant ($P < 0.005$), explaining 17% of the total variance. The regression analysis indicates that the A+ trials revealed a response to the odorant that was far greater than the baseline and B- measurements. This implies that the A+ condition produces excitatory learning, while the B- condition does not.

We also investigated retention of this learned excitatory response to the CS by contrasting the first A+ post-test measurement, taken 6 min after the last conditioning trial, and the second A+ post-test measurement taken 24 h after conditioning (POST1 VS POST2). The non-significant differences between the two post-test measurements clearly demonstrate 24 h retention of the learned discrimination.

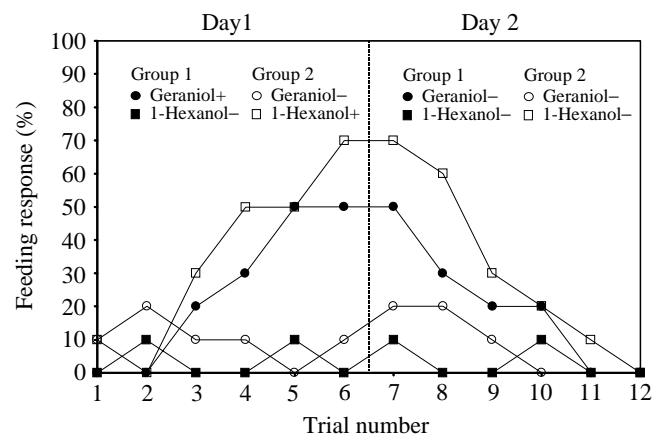


Fig. 5. Measurements of the conditioned cibarial pump muscle reflex to A+ and B- odor presentation during conditioning on day 1 (responses prior to presentation of the unconditioned stimulus, US) and in response to the conditioned stimulus (CS) during extinction trials on day 2. A+ and B- (see text for an explanation) treatments were counterbalanced by odor: geraniol A+ 1-hexanol B- and (N=20) and 1-hexanol A+ and geraniol B- (N=20).

Discussion

We have now established that both male and female *M. sexta* can modify the cibarial pump reflex through prior olfactory experience by using two different non-pheromonal odorants, geraniol and 1-hexanol, both of which produce strong evidence of associative conditioning. We have demonstrated that it is the

Table 3. Results of general linear model and analyses for experiment 2

Variable	NDF, DDF	Type I SS	R SQ	F value	Probability
Model	22	4179.53	0.497	4.36	0.0001**
Error	97	4225.18			
BASELINE VS B-	1, 19	6.07	0	0.04	NS
POST VS B-, BASELINE	1, 19	1439.69	0.171	0.03	0.0051**
POST1 VS POST2	1, 19	1.87	0	0.04	NS
ODOR	1, 19	4.69	0	10.02	NS
INDIVIDUAL DIFF	19, 97	2729.08	0.324	3.30	0.0001**

Regression:

$$\text{Delta frequency} = 0.318 + 7.348(\text{POST VS B-, BASELINE})$$

BASELINE VS B- is a dummy-coded variable that juxtaposes the B- condition and the initial responses to both odors and tests the hypothesis that the B- condition does not produce a change in responsiveness.

POST VS B-, BASELINE is a dummy-coded variable that juxtaposes the post-trained A+ response levels to the initial and the B- response levels.

ODOR is coded 0 for 1-hexanol and 1 for geraniol.

INDIVIDUAL DIFF is a categorical variable representing individual differences.

The regression analysis displays the intercept and the standardized value of β for all significant continuous variables.

NS, not significant. ** $P \leq 0.005$.

For further details, see Table 1.

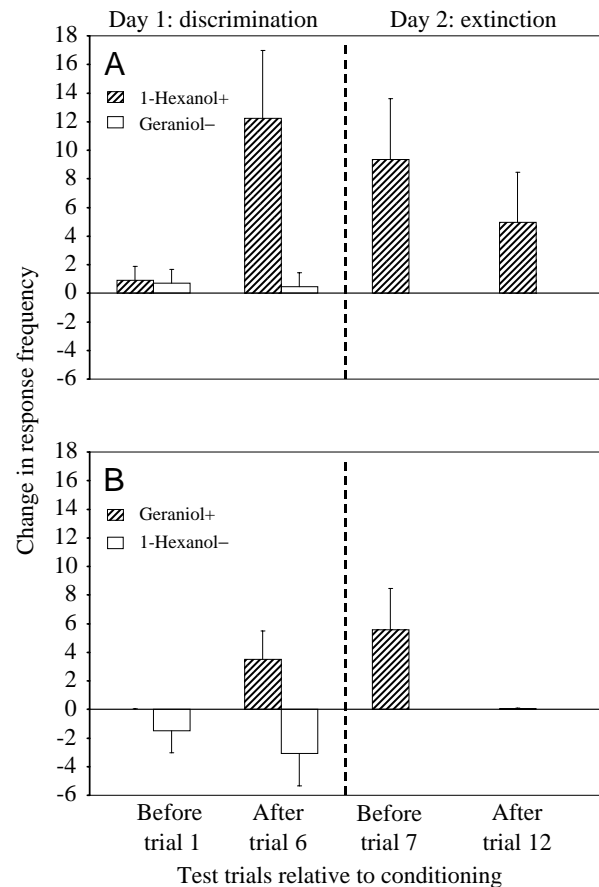
configuration of CS-US contiguity and contingency seen in the forward-paired condition that allows the formation of an excitatory learned response to a previously novel odor, which is indicative of associative and not non-associative processes. Excitatory CS-US associations develop from forward pairing within the first few pairings and are retained for at least 24 h; no other pairing condition used in this study developed such an association. Specifically, when the odorants were backward-paired with sucrose presentation, a negative contingency was imposed on the CS-US relationship, and this condition failed to produce an increase in cibarial pump reflex activity in response to CS presentation.

We observed a high initial response to CS presentation in the forward-paired groups in experiment 1. Although this effect was significant, it does not detract from the central argument that moths in these groups increased their response level over a number of trials as a function of the forward pairing of odor presentation followed by sucrose. Furthermore, in all control groups, subsequent forward pairing of odor then sucrose on the second day also produced similar increases in response strength over trials. We believe, therefore, that this effect is attributable to inter-individual differences.

To ensure that the associative effect was attributable to odor presentation in the forward-paired treatment, and not to other extraneous environmental events such as movements by the

experimenter, which co-vary with sucrose presentation, we implemented the air-paired treatment. This control protocol contained all the features of the forward-paired treatment condition but lacked an odorant (CS). The fact that no detectable learning occurred in this group suggests the

Fig. 6. Mean change in spike activity recorded from the cibarial pump muscle in response to odor presentation prior to conditioning and again after six conditioning trials on day 1 and prior to and after six extinction trials on day 2. This Figure is broken down according to which odor is reinforced; 1-hexanol (A) or geraniol (B) Note that the resultant measurement is zero for geraniol- on day 2 on both test trials, for geraniol+ on day 1 prior to training, and for both test trials for 1-hexanol- and the second test for geraniol+ on day 2. Values are means + S.E.M., $N=20$.



following two points. First, there was no salient stimulus to signal the imminent presentation of sucrose solution. It may be the case that other stimulus events accentuate odor presentation, creating a more salient matrix of stimulus events. But the odorant is necessary to produce a learned response. Second, on the basis of the conclusion that there were no salient extraneous stimulus events, this treatment condition can be likened to a US-only treatment and thus also demonstrates that there were no substantive non-associative effects attributable to sucrose presentation in relation to subsequent odor presentation. This is important because animals will occasionally become aroused by repeated US exposure. This arousal can increase responsiveness to other stimuli, giving the appearance of conditioning when in fact no conditioning has occurred.

In the random-paired treatment group, our approach was to disrupt contingency by presenting the CS and US randomly with respect to one another within a trial. Furthermore, by randomizing the interstimulus interval from +25 to -25 s, we were able to disrupt the contiguity between CS and US. As expected, we failed to observe an increase in response strength on the first day. Unique to this study of moth olfactory learning was the retardation of acquisition observed in the random-paired group on the second day, when odor was forward-paired with sucrose solution. In comparison with the performance of the backward-paired and air-paired groups on day 2, this response decrement is profound, particularly in moths conditioned with geraniol. Moths in the random-paired group learned at the approximately the same rate as moths in the air-paired and backward-paired groups across all trials, suggesting that the putative inhibitory effect was additive. However, note in Fig. 4A that values for the random-paired group climb steadily across all trials, while values for the air-paired and backward-paired groups reach a plateau within a few trials, indicating more rapid learning.

These observations tempt us to speculate that the random-paired scheme used here may have produced conditioned inhibition to the CS. Indeed, Rescorla (1969, p. 92) defines a conditioned inhibitor as a 'stimulus, which through learning comes to control a tendency directly opposite to that of a conditioned exciter'. Our random-paired conditioning protocol clearly provides an opportunity for an inhibitory association to form between the CS and US. However, Rescorla (1969) and Papini and Bitterman (1993) both rightly point out that simply showing the retardation of subsequent acquisition to a previously random-paired CS does not allow us to rule out phenomena such as learned inattention; for that, additional summation tests are needed. While Abramson and Bitterman (1986) have observed latent inhibition, Couvillon et al. (1999) have extensively explored and failed to find conditioned inhibition using intermodal stimulus dimensions in the honeybee. This has also been the case with intermodal blocking in the honeybee (Funayama et al., 1995), while intramodal blocking effects are readily producible (Smith and Cobey, 1994; Smith, 1997; Couvillon et al., 1997). It is likely then that cross-modal integration of sensory input modalities

is either poor or nonexistent in the honeybee and perhaps also in other insects. More extensive analyses of conditioned inhibition using intramodal stimulus dimensions are required to resolve this issue.

Unlike Fan et al. (1997), we observed a reduction in response strength in the forward-paired group on day 1 after a peak response had been attained at around the sixth trial. We have subsequently observed this reduction with an array of other odorants (K. C. Daly and B. H. Smith, unpublished results) but have yet to confirm its cause. One possibility is that motivation decreases as the number of feedings increases, which would produce an associated increase in response latency and/or decrease in response strength. This effect may not have been detected by the analysis of Fan et al. (1997) because they allowed 30 s for a response to occur. Evidence to support this hypothesis comes from our post-test measurements, which recorded responses for approximately 7 s and were not sensitive to this effect.

Alternatively, the observed reduction in response strength may also have been attributable to CS/US habituation. If this is borne out by subsequent analysis, it would imply a dual process of associative learning, on the one hand, which increases response strength, while habituation to either or both the CS and US, on the other hand, produces an increasingly negative effect on response strength with repeated trials. Although this study was not designed explicitly to investigate this class of learning effect, our data from the forward-paired group are consistent with this interpretation. First, considering the acquisition curves for the forward-paired group on days 1 and 2 (Figs 3, 4), at least two characteristics of habituation emerge. On day 1, if learning reaches an asymptote and habituation continues to accrue (to either the CS or US), we would expect a decrease in response strength with additional trials. This appears to occur not only on day 1 but also on day 2, although to a lesser degree. Additional evidence comes from the spontaneous recovery of response strength seen on day 2. Mean changes in response frequencies for the random-paired and backward-paired groups on day 1 also indicate a habituating response. This patterned effect, however, is clear only in the forward-paired group and is only subtly evident in the air-paired and backward-paired groups on day 2.

We find general agreement with Hartlieb (1996) and Fan et al. (1997) and with respect to characteristics of moth olfactory learning such as initial response strength and maximum response strength. Fan et al. (1997) demonstrate retention of learning 120 min post-training, and we have now extended this to 24 h in a moth preparation. Like Fan et al. (1997), we did not observe the sex differences in acquisition rate to odors noted by Hartlieb (1996) in *H. virescens*. This may reflect evolutionary differences in relative reliance on olfaction among males and females of these different species.

The air-paired and backward-paired groups, which did not demonstrate increased cibarial pump reflex activity on day 1, later showed an increase on day 2 when they received forward pairing of odor and sucrose solution. This control measure indicates that the change in responsiveness to odor was not due

to differences among individuals of different groups but that a relationship exists between odor presentation and sucrose presentation when paired in that order; i.e. that sucrose presentation is contingent on odor presentation. Our results show that CS–US contiguity in the forward-paired condition is also needed. The importance of a short inter-stimulus interval has previously been demonstrated in *A. mellifera* (Menzel and Bitterman, 1983) and in *S. littoralis* (Fan et al., 1997), but is not essential for conclusive demonstration of associative processes.

Our data also reveal discrimination learning. We have shown, in a counterbalanced design, that *M. sexta*, like the honeybee (Bitterman et al., 1983; Menzel, 1990), can differentially increase response strength to an odor that was followed by sucrose while simultaneously not increasing response strength to a second odor that was not followed by sucrose. By counterbalancing, we showed that changes in response strength were not due to the odor *per se*, but that the odor was properly paired with reinforcement. Evidence of discrimination learning was also robust in 24 hour post-tests. A differential increase in response strength to the reinforced odor strongly suggests not only that the moths are able to discriminate between the odors but also that they changed their responses to an odor on the basis of whether it was reinforced; this effect can only be explained in terms of associative processes.

M. sexta does not demonstrate proboscis extension behavior upon presentation of sucrose on the antenna. We have therefore developed and validated a different training protocol, which reliably produces learning, is directly comparable with PER training, is readily adaptable to other nectar-feeding insects and has some distinct methodological advantages. First, because behavioral observations were based on EMG recordings, precise quantitative measurements could be made. Smith and Menzel (1989) point out that feeding behavior in insects is not simply the extension of the proboscis, but that many sub-behaviors are involved. EMG measurements can be employed to focus on one aspect of a ‘feeding motor program’ and describe it more accurately. Observational data, however, being inherently more categorical and subjective, lack the quantitative resolution obtained from EMG recordings. This study clearly demonstrates the clarity with which EMG monitoring of cibarial pump reflex activity can index learning in *M. sexta*. A second advantage is that other EMG-based analyses, such as timing effects, latency of response, spike amplitude and spiking duration, are possible using the same basic technique, thus offering different measurement resolutions to tackle issues in learning theory. This precision measurement model will lend itself particularly well to psychopharmacological analysis of learning, in which pharmacological applications can have either very subtle or radical effects on learning and behavior.

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