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Olfactory-based discrimination learning in the moth, *Manduca sexta*

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Abstract

Moths possess highly tuned olfactory capabilities, which can detect very low concentrations of pheromonal odorants. Much is known about the structure and function of the moth olfactory system with respect to detection of pheromones. However, we lack an understanding of the broader olfactory system, in particular, to what degree are moths capable of detecting and discriminating odorants that are not components of pheromone blends. Here we describe a methodology used to investigate the discriminability of nonpheromonal odors in moths. In a series of experiments we show that the moth *Manduca sexta* can (1) discriminate a number of different odors but (2) that methyl jasmonate, neither readily conditions to a food reward nor is it readily discriminated from another odor. The lack of a response to methyl jasmonate may be related to its role in host plant defense. This work provides a basis for future mapping of physiological and pharmacological studies of nonpheromonal coding in insects onto learned behavioral responses to those odorants. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Studies of several species of moths have begun to reveal the physiological bases that underlie behavioral responses of males to female sex pheromones (Hildebrand and Shepherd, 1997). Once detected by males, these odors, which are usually species-specific blends of two or more components, elicit directed upwind flight that culminates in mating (Kennedy et al., 1981; Vickers and Baker, 1994). Transduction and interpretation of information about the components of the blend and the temporal characteristics of the odor plume takes place in primary sensory cells of the antennae and in the first-order interneurons of the antennal lobes of the brain (Christensen and Hildebrand, 1987a; Waldrop et al., 1987; Christensen et al., 1998). This early processing constitutes a narrowly tuned subsystem within a male moth's olfactory system. Presumably this dedicated labeled-line subsystem should evolve

because of the reliability of the pheromonal signal over evolutionary time (Smith, 1996).

Additionally, moths like *Manduca sexta*, for example, also respond to an array of non-pheromonal plant odors. These odors may not be as reliable over time as sex pheromones in the sense that the presence, amount and blend ratio can vary over hours or seasons but which nevertheless elicit stereotypic, or innate, behaviors. While data exploring physiological bases for non-pheromonal responses in moths exists (e.g. Anderson et al. 1993, 1995; Anton and Hansson, 1994; Jonsson and Anderson, 1999) this area is still largely unexplored. In the case of oviposition on host plants, there appears to be odorants emitted from larval frass (Anderson et al., 1993) and plant odors (Anderson et al., 1995) that act to influence female moths ovipositing behavior. In these cases, there appears to be appropriate specializations of the olfactory system that are analogous, at least in terms of tuning properties, to the sex pheromone subsystem.

It is well known that *M. sexta*, like many adult insects, regularly feeds on floral nectar, though the specifics of this are poorly documented in moths. Given the ephemeral nature of floral odors relative to the lifetime of an individual moth, it is not surprising that recent studies

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of several moth species have revealed the capacity for moths to learn the relationships between odor and sucrose reinforcement (Hartlieb, 1996; Fan et al., 1997; Daly and Smith, 2000), which parallels that well-established ability in the honey bee (Menzel and Bitterman, 1983).

Given too, the accessibility of the moth olfactory system to physiological analysis, it would be enlightening to investigate responses to odors other than sex pheromones. At the very least this would provide the possibility of comparing specializations within the olfactory system of the same species for meeting the demands of behavioral responses to signals that differ in reliability over time. Studies of sex pheromone processing have been driven by the elegant, stereotyped behaviors that are innately elicited by pheromones. With other types of odors, particularly plant odors, there may not be clear innate behavioral programs that are initiated by presentation of the odor. Therefore it is necessary to first develop conditioning protocols that associate a target odor with reinforcement (Smith and Getz, 1994), as has been done for the honeybee (Smith and Menzel, 1989). Once developed, these behavioral protocols can be used to establish how non-pheromonal odors are represented in the olfactory system, which is useful for development of testable physiological hypotheses concerning olfactory processing.

Along this line, two preparations have currently been employed to demonstrate Pavlovian conditioning in three moth species including *Spodoptera littoralis* (Fan et al., 1997) *Heliothis virescens* (Hartlieb, 1996) and *Manduca sexta* (Daly and Smith, 2000). The procedure for conditioning *S. littoralis* and *H. virescens* is similar to that which has been used for many years to condition honeybees (Menzel and Bitterman, 1983). Briefly, the conditioning stimulus (CS), in this case an odor, is presented to the antennae of the animal, which is immediately followed by application of an unconditioned stimulus (US), sucrose solution, to the antennae. This elicits a reflexive extension, or unconditioned response (UR), of the proboscis, an appetitive feeding response. In as little as one of these odor–sucrose pairing trials, honeybees have been shown to elicit a conditioned response (CR) by extending its proboscis to the odor alone. *M. sexta*, unlike *S. littoralis* and *H. virescens* and the honeybee, does not reflexively extend its proboscis in response to presentation of sucrose solution upon the antennae. This is likely due to the fact that these moths hover above flowers they feed upon, thereby eliminating the need for taste receptors on the antennae. Therefore, in *M. sexta* the cibarial pump muscle response (not proboscis extension) is conditioned to odor by presentation of sucrose solution directly upon the proboscis immediately following odor presentation. The responses of these muscles are monitored via electromyograph (EMG) rec-

ordings prior to, during and following conditioning (Daly and Smith, 2000).

These studies now need to be extended through use of generalization and discrimination conditioning protocols, because these are important experimental paradigms for investigating how stimuli are perceptually represented in the brain (Mackintosh, 1983). Discrimination learning is, in the present case, a product of differentially reinforcing one stimulus, in this case an odor, while in tandem not reinforcing a second odor. Stimulus generalization on the other hand is the degree to which a novel stimulus elicits the conditioned response, based on its similarity to the stimulus used in the prior formation of the conditioned response. Discrimination and generalization experiments have been used, for example, to investigate the discriminability of subtly different monomolecular odorants based on physical characteristics such as carbon chain length (Smith, 1993) thereby providing information about which stimulus dimensions are relevant to odor coding in olfactory systems. Furthermore, pharmacological investigations have begun to unravel the complex relationship between physiological representations for odors and discriminability in the honeybee (Stopfer et al., 1997; Hosler et al., 2000).

Here we extend previous work showing discrimination learning to several pairs of odors by *M. sexta* males and females. In large part moths show a well-developed ability to discriminate floral- and/or plant-related odors. We found only one exception in the case of methyl jasmonate, where learning appeared to be disrupted. In retrospect this particular odor may have a biological meaning because of the direct role it plays in plant defense (Karaban and Baldwin, 1997) and its implication as a kairomonal attractant of natural predators of *M. sexta* (Turlings et al. 1990, 1995).

2. Methods

Male and female *Manduca sexta* were obtained during middle and late-stage pupal development from Arizona Research Labs, Division of Neurobiology via overnight delivery. Rearing conditions have been described in detail elsewhere (Bell and Joachim, 1976). Upon arrival, pupae were classified by sex then individually placed in brown paper bags where they remained undisturbed until used in an experiment. Bagged pupae were stored in environmental chambers that held temperature at 28°C, 80% relative humidity, with a 16/8 L/D cycle.

Pupae were checked once daily, just before initiation of the dark cycle. An eclosion date was recorded on bags in which newly emerged pupae were found. Age at initiation of training was between 5–7 days post eclosion. This holding period, without access to food or water, was found to increase motivation while not significantly damaging the subjects such that they had dif-

faculty performing the task (Daly and Smith, 2000). Subjects were randomly assigned to one training group and used only once.

2.1. Preparation

The general methodology described below has been implemented successfully in prior studies (Daly and Smith, 2000) and was originally adapted from the proboscis extension response (PER) conditioning protocol in the honeybee (Menzel and Bitterman, 1983). Briefly, subjects were restrained in individual plastic preparation tubes. The proboscis was then extended and threaded through a 4 cm length of flexible surgical tubing leaving 1–3 cm of the distal end of the proboscis exposed. The tubing was then affixed to the front of the preparation tube with a soft wax. A Teflon-coated fine silver wire electrode was placed just under the surface of the head capsule of the subject, 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 contra-lateral compound eye. The leads from the preparation tube were plugged into a multi-port magazine used to train multiple subjects. Leads from the magazine connected to an A-M Systems model 1700 differential AC amplifier. Output from this amplifier lead to an oscilloscope, an audio output device and to an A/D board in a computer.

An odor cartridge was made from a 1 cc tuberculin glass syringe in which 3 μ l of odorant was placed on a strip of filter paper. Separate syringes were used for each odorant to avoid cross contamination. The odorant cartridge was positioned in the front of the training stage and connected to an aquarium pump air supply. A computer controlled shunt controlled airflow through the odor cartridge. Airflow through the odor cartridge was measured at 7.5 ml/s. At the back of the training stage an exhaust port evacuated odorant from the training area. For each trial, subjects were placed in the center of the stage such that the odor cartridge was aimed directly at the subject's head from approximately 9 cm away creating a dispersion field wide enough to cover both antennae; this has been confirmed with titanium tetrachloride (liquid smoke) tests. When the shunt was opened, odorant was gently blown over the subject's head and antennae and then drawn into the exhaust vent. The time for the odor to reach from the odor cartridge to the antennae has been estimated from antennal lobe recordings using the same configuration to be about 200 ms (personal observation). For all experiments subjects received one exposure to one odorant (CS) per trial. Odorants, whether reinforced with sucrose solution (CS+) or not (CS–), were presented for 4 s during each conditioning trial. In cases where the CS was forward-paired with a sucrose US, the sucrose was presented for

4 s, starting 3 s into the CS presentation, producing a 1 s overlap.

2.2. Response measures

Response measures were based on changes in the rate of electromyographic (EMG) activity recorded from the cibarial pump muscle, a procedure previously described by Daly and Smith (2000). First, subjects were scored based on a detected change in feeding behavior upon presentation of the CS. This score was based on three indicators: sound from the audio output device attached to the amplifier; visualization of the EMG record during a trial on the oscilloscope and extension of the tip of the proboscis that protruded from the end of the plastic tube. If the experimenter observed an increase in activity within 3 s subsequent to CS onset, a response was recorded for that trial. These observational data were used descriptively to show the acquisition of learned response as it developed.

Electrical activity of the cibarial pump muscle (EMG) was digitized at a 2.5 kHz sampling rate and stored on disk. Each recording was 10 s in duration and sampled activity for approximately 3 s before odor presentation, 4 s during odor presentation, and approximately 3 s after termination of odor presentation.

2.3. Differential conditioning

A total of 80 subjects were conditioned in one of four discrimination-training groups. For the first three groups cyclohexanone (Aldrich Chemical Co, 99.8% purity) was paired with methyl salicylate (Sigma Chemical Co, 99% purity), methyl jasmonate (Aldrich Chemical Co, 95% purity) or geraniol (Sigma Chemical Co, 98% purity). In the fourth group, geraniol was paired with 1-hexanol (Aldrich Chemical Co, 98% purity). All odors were presented undiluted in 3 μ l dosages. None of the odorants are known pheromone components in *M. sexta*. In addition, cyclohexanone to our knowledge is a synthetic compound, making it a biologically novel stimulus, that is, it is unlikely that *M. sexta*, in particular, has encountered this compound throughout its natural history.

Each pair of odors was differentially reinforced in a counterbalanced design. So for example in one group, a sub-group of 10 subjects received cyclohexanone reinforced with sucrose (CS+) and methyl salicylate without sucrose reinforcement (CS–). The other sub-group of 10 subjects received cyclohexanone CS– and methyl salicylate CS+. The CS+ odorant was forward paired with 5 μ l of a 1.25 molar sucrose solution while the CS– odor was presented alone. Each subject received eight conditioning trials (4 CS+ and 4 CS–) in a pseudo-randomized fashion counterbalanced by type of trial (CS+ or CS–). The patterns of pseudo-random

presentation used were as follows AB–ABBABAABA–AB–AB and BA–ABBABAAB–BA–BA where A represents the CS+ condition and B represents the CS– condition. Note that the pre and post-tests, separated from conditioning trials by dashes, were reversed as a counterbalancing measure.

A 6-min inter-trial interval was maintained from trial to trial irrespective of whether animals received CS+ or CS– trials. Observational data were collected for all conditioning and test trials. In addition, cibarial pump muscle activity was collected in response to presentation of the CS+ and CS– odors without reinforcement at three points in the conditioning procedure: baseline measures were collected 6 min prior to conditioning; post-test measures were collected at 6 min after conditioning and again 24 h later. These test measures provided evidence of discrimination learning and retention. Test trial sequences were also counterbalanced by trial type to control for sequencing and stimulus generalization effects.

2.4. Odor intensity

Results of experiment 1 indicated that methyl jasmonate was either not salient as a CS, or that moths, for possibly biological reasons, could not acquire a conditioned feeding response to it. Previous research by Bhagavan and Smith (1997) on the effects of stimulus intensity on discrimination learning indicate that if one odorant is substantially more intense than another, honeybees will encounter difficulty in learning the weaker of the two. Additionally, Daly and Smith (2000) have shown in this preparation that forward pairing with a blank produces no substantive learning. Hence, we hypothesized that if the strength of the more intense odor (cyclohexanone) is reduced, then learning to the weaker odorant might be more evident. Alternatively, lowering the intensity of cyclohexanone may not increase subsequent salience, or perceived intensity, of methyl jasmonate, indicating either that *M. sexta* has difficulty detecting this odor or that methyl jasmonate may already hold some other conflicting biological meaning. To address this issue we first attempted to identify a concentration of cyclohexanone that was lower yet capable of producing a learning effect when forward paired with sucrose. 40 subjects were placed into four groups of 10 subjects each. All four groups received 10 forward-paired trials of odor and 1.25 molar sucrose solution. Groups 1–3 received odor from cartridges prepared with 3 μ l of 3%, 0.03% and 0.003% concentrations cyclohexanone in acetone respectively, the fourth group received of methyl jasmonate (3 μ l pure). Observational data were collected to create acquisition curves for each group. From this we selected the concentration of cyclohexanone that produced an acquisition curve that most closely matched that produced with methyl jasmonate.

2.5. Generalization of conditioned response

We also performed a generalization analysis to further elucidate the lack of discrimination learning in the cyclohexanone-/methyl jasmonate+condition. In this experiment two groups of male and female moths received 10 forward paired conditioning trials of either cyclohexanone and sucrose or methyl jasmonate and sucrose. The same general forward pairing protocol was used as that described in experiment 2. Twenty four hours after training, moths were post-tested, exposing them to both odors with a 30-min interval between each of the two trials and counterbalancing the order of odor presentation within the groups. Cibarial pump muscle response was recorded (as previously described) in response to presentation of each odor.

2.6. Data analysis

A conditioned response was recorded during conditioning trials if the subject exhibited increased cibarial pump activity during the 3 s period from the initiation of CS presentation until the initiation of US presentation. These data were used to create acquisition curves, which show the proportion of subjects displaying a conditioned response by trial and \pm odor combination.

Daly and Smith (2000) show representative raw EMG traces in response to odor presentation prior to and after conditioning and detail procedures for spike counting. Briefly, spike counts of each raw EMG record were performed using a spike counting application created in HP-VEE. Spike counts were collected in two time epochs: one prior to the onset of the CS, and one from CS onset until the end of the trial. Spike counts were converted to pre-CS and post-CS frequencies. A ‘ Δ frequency’ score created for each test trial by subtracting the pre-CS frequency from the post-CS frequency. This conversion cancels out effects associated with sensitization and/or changing levels of baseline activity, which change as a function of prior sucrose presentation thereby rendering a clarified CR measure. Thus, only an increase in spike activity during odor presentation registered as a non-zero response. For all statistical analyses the Δ frequency scores were rank ordered for within-group comparisons of differences in conditioned response (CR) using the Wilcoxon inverted U tests for nonparametric data (SAS, 1996). Univariate *t*-tests of means (SAS, 1996) were performed to assess whether mean responses were significantly different from zero.

3. Results

3.1. Differential conditioning

Fig. 1 displays the initial cibarial pump muscle response to presentation of each odor prior to condition-

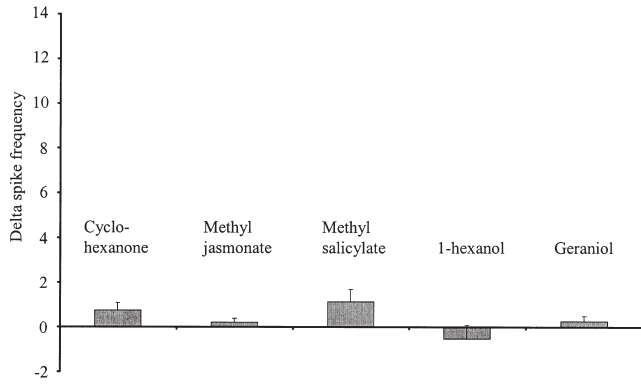


Fig. 1. Mean Δ spike frequency of the cibarial pump muscle in response to the first odor presentation for each odor used in the study. Means represent initial response by odor across all groups where that odor was reinforced. Because odors were used to varying degrees, the means and standard errors displayed for each are based on different N : cyclohexanone $N=100$; methyl jasmonate $N=60$; methyl salicylate $N=20$; 1-hexanol $N=20$; geraniol; $N=40$.

ing. For this figure we have averaged the initial response to each odor across all groups for which the odor was used. Univariate t -tests of the mean initial response by group showed no means significantly different from zero, suggesting that there was little initial response to any of the odors used. We then compared this baseline response to the initial post-test (6 min after conditioning) and the 24 h post-test, for the non-reinforced, or CS $-$, odors using a Wilcoxon's inverted U test. This analysis was performed both on initial and 24 h post-tests individually and on the average of these scores. Irrespective of whether we analyzed post-tests separately or as an average, we found that there were no significant differences between the initial response strength and the response rates for the post-tests when the odor was not reinforced with sucrose. This indicates that responses to non-reinforced odors do not change.

For the reinforced odors, comparisons were made between the initial and 24 h post-tests. We found no significant differences, indicating that moths did retain learning to the reinforced odor for at least 24 h. Therefore we averaged the post-test scores for subsequent analysis and focused on differences between reinforced and non-reinforced odors.

Cibarial pump response to the CS $+$ and CS $-$ odors after conditioning for the first experiment are displayed in Fig. 2 by odor pair. Notice in Fig. 2(a–c) that the reinforced odor produces a greater response than the non-reinforced odor. Note too that the responses to the non-reinforced odors are comparable to the initial response magnitude that these odors elicited (Fig. 1). In Fig. 2(d) however, response to cyclohexanone, when reinforced, increased in a characteristic fashion while when methyl jasmonate was reinforced it did not. Table 1 statistically confirms these differences in learned

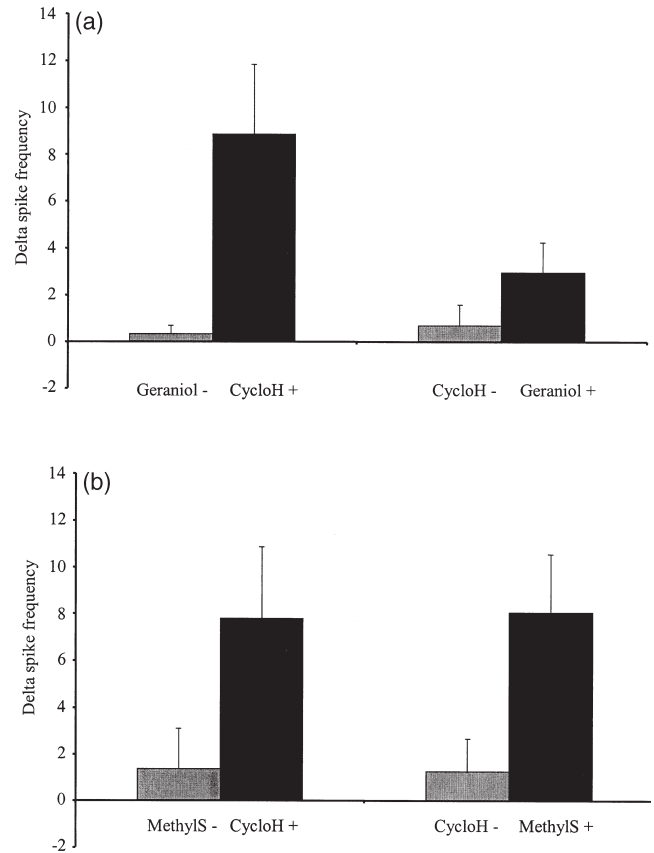


Fig. 2. Mean Δ spike frequency of the cibarial pump muscle in response to odors that were differentially reinforced in a counterbalanced design. While moths within each group experienced both odors for an equal number of trials, presented in a pseudo-randomized order, only the odor demarked with a '+' was followed by a sucrose solution reinforcement. (A) When geraniol (+) is reinforced it elicits a response that is higher than cyclohexanone. However when cyclohexanone (+) is reinforced the pattern of response is reversed. The same is true of cyclohexanone and methyl salicylate (B) and of geraniol and 1-hexanol (C) but not of cyclohexanone and methyl jasmonate (D). In the latter case, when cyclohexanone (+) was followed by sucrose solution and methyl jasmonate (-) was not, the predicted pattern of response occurs. However, when methyl jasmonate (+) was reinforced and cyclohexanone (-) was not, no evidence of discrimination emerged.

response between the CS $+$ and CS $-$ odors mentioned above.

3.2. Odor intensity

Results of the dilution series for experiment 2 are displayed in Fig. 3. The goal of this experiment was to elucidate a concentration of cyclohexanone that produced acquisition that most closely matched that produced by methyl jasmonate. Since the 3% cyclohexanone dilution produced acquisition nearly identical to methyl jasmonate, it was selected for use in experiment 3.

Fig. 4 displays differential response strength for methyl jasmonate and 3% cyclohexanone. Here we see

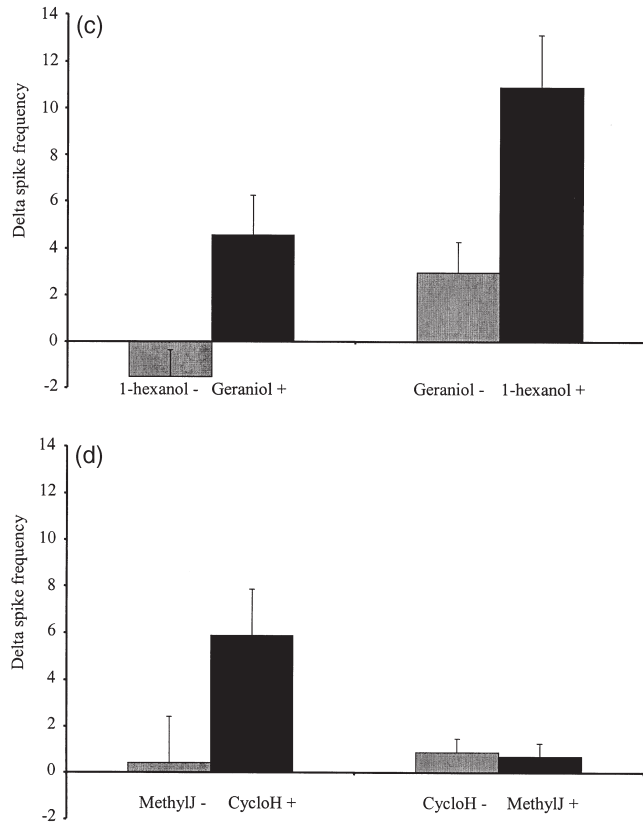


Fig. 2. (continued)

that reducing the salience of cyclohexanone only served to reduce the response strength to that odor when forward paired. Reduction in the salience of cyclohexanone did not have the effect of increasing the response strength to methyl jasmonate (+). This indicates that at

these concentrations, subjects are not able to readily discriminate the two odors. Results from statistical analysis (Table 2) confirm that correcting for concentration did not affect the discriminability of the two odors.

3.3. Generalization of conditioned response

Results of the odor intensity experiments prompted us to investigate the generalization of learned response between methyl jasmonate and 3% cyclohexanone. Fig. 5 displays mean Δ frequency in response to both odors by pre-conditioned odor. The general pattern of generalization from a preconditioning odorant to a novel one is consistent with experiment 1. That is moths conditioned to cyclohexanone respond to it but not methyl

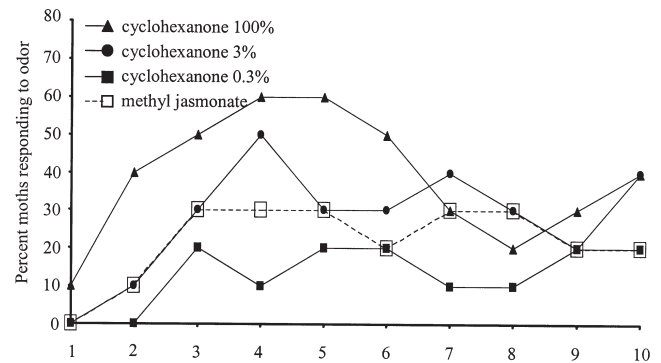


Fig. 3. Acquisition of conditioned response as measured by an increase in EMG activity of the cibarial pump muscle during the 3 s between odor onset and sucrose reinforcement. Shown are acquisition curves for methyl jasmonate and three concentrations of cyclohexanone. Note that a 3% concentration of cyclohexanone produces acquisition most closely to that produced by methyl jasmonate.

Table 1

Results of Wilcoxon's ranked tests for differences in Δ frequency between + and - odors for experiment 1^a

± Odor pairs	N	Mean under HO	SD under HO	Z	Probability
Cyclohexanone +	10	27.20	34.19	3.90**	0.0001
Geraniol -		13.80			
Geraniol +	10	24.55	32.15	2.50*	0.0117
Cyclohexanone -		16.45			
Cyclohexanone +	10	27.30	34.19	3.96**	0.0001
Methyl-jasmonate -		13.70			
Methyl-jasmonate +	10	22.30	22.96	1.55	ns
Cyclohexanone -		18.70			
Cyclohexanone +	10	27.00	33.27	3.89**	0.0001
Methyl-salicylate -		14.00			
Methyl-salicylate +	10	25.97	33.76	3.23**	0.0012
Cyclohexanone -		15.03			
Geraniol +	10	26.17	31.49	3.58**	0.0003
1-Hexanol -		14.83			
1-Hexanol +	10	25.57	33.7	2.99**	0.0026
Geraniol -		15.42			

^a Moths were differentially reinforced in relation to two odors. Odors followed by a '+' sign were forward paired or reinforced with sucrose solution while odors followed by a '-' sign were not followed by reinforcement. Pairs of odors were pseudo-randomly presented in the above counterbalanced pairs. * $P < 0.05$; ** $P < 0.001$.

Table 2

Results of Wilcoxon's ranked tests for differences in Δ frequency between + and – odors for experiment 2

	<i>N</i>	Mean under HO	SD under HO	<i>Z</i>	Probability
Methyl-jasmonate +	20	38.30	87.11	0.08	ns
3% Cyclohexanone –		38.69			
3% Cyclohexanone +	20	41.70	75.98	0.63	ns
Methyl-jasmonate –		39.30			

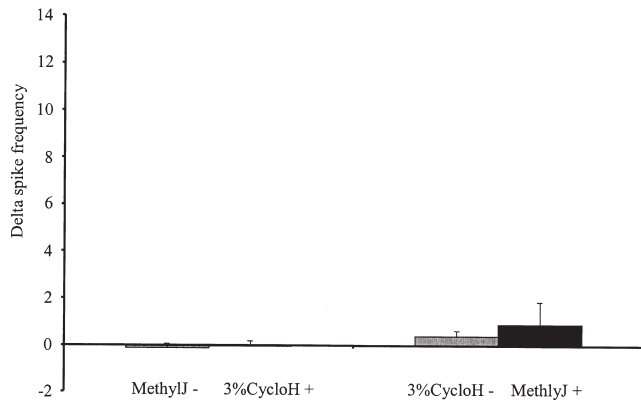


Fig. 4. Mean Δ spike frequency of the cibarial pump muscle in response to methyl jasmonate and a cyclohexanone diluted to 3% in a counterbalanced design. Here, when cyclohexanone concentration is reduced there is little evidence of acquisition or discrimination.

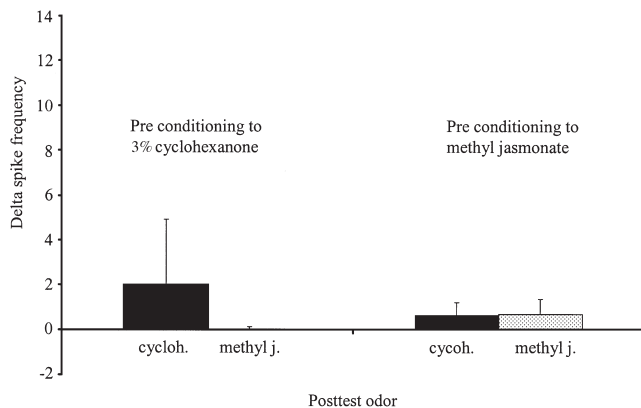


Fig. 5. Mean Δ spike frequency of the cibarial pump muscle indexing conditioned response to the conditioning odor and generalization of response to the novel odor. Note that when moths were conditioned to 3% cyclohexanone they show a conditioned response to it but that response did not generalize to methyl jasmonate. However, when conditioned to methyl jasmonate moths show little response to either.

jasmonate while moths conditioned to methyl jasmonate show little conditioned response to either odor. While these observations are consistent with experiment 1, bivariate comparisons of posttest CR between the trained

odor and non-trained odor (Table 3) yielded no significant differences between response strength to either odor, irrespective of which odor was paired with sucrose reinforcement. Non-significant results indicate that the moths CR to the trained and non-trained odors were not significantly different, or there is a high degree of generalization between these two odors at these concentrations.

4. Discussion

We have described discrimination learning in *M. sexta* using changes in feeding muscle activity as an index of acquisition, discrimination and retention. This methodology uses differential reinforcement of two odors in a Pavlovian fashion where one odor is forward paired with an excitatory US and the other is presented alone in a series of pseudo-random conditioning trials. The forward pairing of odor and sucrose solution produces an excitatory feeding response that is dependent on the reinforcement, independent of the odor used. Additionally, the consistent presentation of odor alone fails to augment any initial responsiveness that an odor might produce.

For example, when cyclohexanone (+) was paired with sucrose reinforcement and methyl salicylate (–) was not, an excitatory CR was produced when cyclohexanone was presented alone in post-tests. The response to methyl salicylate did not change. However, when methyl salicylate was followed by sucrose solution and cyclohexanone was not, a CR developed in relation to methyl salicylate presentation and there was relatively little or no response to cyclohexanone. Thus discrimination of these two odors was symmetric. In addition, moths showed a general ability to discriminate between geraniol and 1-hexanol, and between geraniol and cyclohexanone.

These results suggest that there is a change in relationship between the reinforced odor and its underlying sensory-processing subsystem and neural centers that are activated by reinforcement. This increase cannot be due to a general arousal produced by the sensitizing qualities of the US, because the increase was specific to the CS+. In contrast, the relatively low response levels to the CS–

Table 3

Results of Wilcoxon's ranked tests for differences in Δ frequency between trained and novel odors for experiment 3

Training odor	N	Mean under HO	SD under HO	Z	Probability
3% Cyclohexanone	20	19.55	28.11	0.65	ns
		21.45			
Methyl-jasmonate	20	21.40	22.97	0.76	ns
		19.60			

could be consistent with a number of interpretations. There is likely little or no appetitive associative strength between that odor and reinforcement, because the latter is presented on different trials (and in relation to a different odor) that are well separated from the trials during which the CS— was presented. However, at the present level of analysis, the decreased response to the CS— odors is also consistent with at least three other explanations. Non-reinforced presentation of a CS may produce habituation (Thompson and Spencer, 1966) of any innate response to that CS. Furthermore, non-reinforcement may also result in latent inhibition (Mackintosh, 1983) or conditioned inhibition (Rescorla, 1969; Pappini and Bitterman, 1993). None of these alternatives can be ruled out at this time.

We have also identified a pair of odors that moths do not discriminate well. In the present experiment we found that methyl jasmonate was not discriminable from cyclohexanone when the former was forward paired with an excitatory US and cyclohexanone was not. However, when cyclohexanone was forward paired with an excitatory US there was clear evidence for discrimination. Separate forward pairing of odors individually (see Fig. 3) produced acquisition to both odors over 10 forward-paired trials (four more forward-paired trials than in our discrimination experiment), although we found that the moths generally demonstrated a relatively weak conditioned response to methyl jasmonate. These results suggested that cyclohexanone may be significantly more salient than methyl jasmonate. Indeed differential intensity between odors has been shown to affect generalization and discriminability of conditioned responses in the honeybee (Bhagavan and Smith, 1997), in a manner consistent with our methyl jasmonate data.

Alternatively, methyl jasmonate may have an innate meaning to the moth, which actively disrupts our learning paradigm. Activation of an innate response pattern may interfere with acquisition and/or expression of learned behavior. Smith (1993) proposed a similar interpretation for conditioning of alarm pheromone in honeybees. Honeybee workers can be conditioned to exhibit appetitive feeding responses to alarm pheromone, but the response levels are typically less strong than after equivalent conditioning of other odorants. Hartlieb et al. (1999) have also shown in *S. littoralis* that the biologically relevant blend of female sex pheromones disrupted

acquisition of a conditioned proboscis extension more so than individual components of the blend, which by themselves do not appear to be a relevant pheromonal cue. In two separate experiments we attempted, therefore, to clarify this issue. Our first approach was to investigate the effect of lowering the intensity of cyclohexanone by diluting it until we achieved an acquisition rate that closely matched that produced by methyl jasmonate and then replicating our initial discrimination experiment. We found that lowering the concentration of cyclohexanone to a 3.0% concentration did not yield statistical evidence of discrimination and only served to disrupt learning of both odors. Thus when cyclohexanone is at high concentrations, moths can discriminate it from methyl jasmonate but when cyclohexanone is presented at this low concentration moths have difficulty discriminating these two odors and hence have difficulty developing an appetitive feeding response to either. Thus, at least assuming that intensity is related to perceptual salience, the reduction in salience of cyclohexanone did not elevate the response to methyl jasmonate suggesting that the problem was not one of differential intensities.

If these two odors at these concentrations were perceptually similar, then the conditioning the moths experienced might be equivalent to partial reinforcement, in which a CS is reinforced on a fraction of the total trials. This pattern of reinforcement typically produces lower levels of conditioned responding, which might account in part the poor learning we observed. Indeed, if the moths perceived these odors as the same then they experienced a 6:8 ratio of reinforced to non-reinforced trials (keeping in mind pre-testing of each odor) before the first post training measure of conditioning. We therefore sought to eliminate this methodological confound by conditioning to one odor then post-testing to both. The results of the generalization experiment generally support the conclusion that there is poor discriminability of these two odors when cyclohexanone is at the lower concentration. However, while not significant, the pattern of responses in this experiment is consistent with the results of the first experiment showing that there is a greater degree of discriminability of odors when the conditioning odor is cyclohexanone. In summary, these data suggest that perhaps the development of an appetitive response to methyl jasmonate is being disrupted.

One possibility is that the methyl jasmonate signal may appear cryptic to *M. sexta*. Although weak, we have shown here that these moths can be conditioned to methyl jasmonate, suggesting that this is not a reasonable explanation. A more intriguing, though speculative explanation regards the potential use of methyl jasmonate as a kairomone. Methyl jasmonate is involved in direct and indirect defense of host plants of *M. sexta* in at least two ways. First, it is involved in the production of proteinase inhibitors (Farmer et al., 1992), which make the plant more difficult for moth larvae to digest. Second, methyl jasmonate is believed to act as a kairomone to signal parasitoids and predators of herbivores like *M. sexta* when a plant is being fed upon (Turlings et al. 1990, 1995). Given our results it might also be the case that methyl jasmonate is related to a hard-wired response in moths like *M. sexta* and that this innate response interferes with acquisition in our paradigm. A female that is able to selectively avoid oviposition upon plants that are emitting methyl jasmonate would produce a 3-fold advantage for her offspring. First, they would avoid direct competition for food from established populations of larvae, which initiated the jasmonic response by feeding on the plant. Second, larvae would avoid exposure to parasites attracted to the damaged plant. Third, larvae would avoid plants that are producing chemical defenses that disrupt the larvae's normal digestive activity.

While we recognize that this a speculative proposition, the fact that *M. sexta* can detect methyl jasmonate but display a disrupted ability to modify behavior to it suggests to us that they may already have a biologically prepared response to this odor, which interferes with appetitive conditioning. If it were the case that methyl jasmonate played a role in deterring females from approaching and ovipositing then there may likely be a sexually dimorphic response to this odor. While the data presented here was not appropriate for this analysis we believe that potential sex differences to methyl jasmonate should be explored in detail.

In conclusion, these data provide a basis for application of established physiological (Christensen and Hildebrand, 1987b) and molecular (Waldrop et al., 1987; Nighorn et al., 1998) techniques to investigation of non-pheromonal processing in *M. sexta*. Ultimately it will allow for comparison of mechanisms involved in encoding a diverse array of odor types (floral odors, pheromones and potential kairomones) to establish the generality or specificity of the olfactory system for processing all of those types of odors in the same species.

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