Characterizing Psychophysical Measures of Discrimination Thresholds and the Effects of Concentration on Discrimination Learning in the Moth *Manduca sexta*

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Abstract

What is the spatial and temporal nature of odor representations within primary olfactory networks at the threshold of an animal's ability to discriminate? Although this question is of central importance to olfactory neuroscience, it can only be answered in model systems where neural representations can be measured and discrimination thresholds between odors can be characterized. Here, we establish these thresholds for a panel of odors using a Pavlovian paradigm in the moth *Manduca sexta.* Moths were differentially conditioned to respond to one odor (CS+) but not another (CS−) using undiluted odorants to minimize salience-dependent learning effects. At 24 and 48 h postconditioning, moths were tested for the presence of a conditioned response (CR) with a blank, then the CS+ and CS− (pseudorandomly) across a 5-log step series of increasing concentration. Results identified discrimination thresholds and established that differential CRs to the CS+ and CS− increased with stimulus concentration. Next, 3 separate groups of moths were differentially conditioned at either one-log step below, at, or one log step above the identified discrimination threshold. At 24 and 48 h postconditioning, moths were tested sequentially with a blank, the concentration used for conditioning, and then undiluted odor. Conditioning at one log step below the discrimination threshold established a CR, indicating both stimulus detection and learning, but was insufficient to establish evidence of discrimination. Moths conditioned at the discrimination threshold were able to discriminate but only when stimulated with undiluted odors, indicating learning, but discrimination measures were hampered. When conditioned above the discrimination threshold, moths had no difficulty in discriminating. These results establish methods for psychophysical characterization of discrimination and indicate that differential conditioning at lowered concentrations biases threshold measures.

Key words: differential conditioning, discrimination threshold, *Manduca sexta*, olfaction, olfactory coding, psychophysics

Introduction

Behavioral studies of olfactory function establish that the ability of animals to discriminate across a broad array of odorants, blends, and concentrations is remarkable. Insects such as moths (Hartlieb et al. 1999; Daly and Smith 2000; Fan and Hansson 2001; Skiri et al. 2005) and honeybees (Smith et al. 1991; Smith and Cobey 1994; Chandra and Smith 1998; Hosler and Smith 2000; Wright et al. 2002; Wright and Smith 2004; Wright, Lutmerding et al. 2005; Wright, Thomson et al. 2005) appear to be no exception in this regard. To account for this seemingly limitless ability with relatively few receptor types, it has been argued that the initial spatial pattern of input is locally transformed within the insect antennal lobe (AL) or vertebrate olfactory bulb (OB) into a spatiotemporal code (Laurent 1999). The ensuing temporal evolution of odor-dependent output from the AL or OB can span from less than 250 ms (Friedrich and Laurent 2001;

Daly, Wright et al. 2004; Brown et al. 2005) to several hundreds of milliseconds (Galizia et al. 2000; Friedrich and Laurent 2001). This temporal evolution may also be organized by subthreshold oscillatory events (Laurent and Davidowitz 1994; Laurent and Naraghi 1994; Laurent et al. 1996; Wehr and Laurent 1996; Stopfer et al. 1997).

Several fundamental questions remain concerning spatiotemporal responses from the AL and OB at the psychophysical limits of an organism's ability to detect or discriminate odors. For example, are spatiotemporal representations within the AL or OB the same at the concentration needed to detect versus discriminate odors? Do these representations become more or less temporally complex with increasing concentration? What percentage of these systems needs to be activated to produce a perceptually salient olfactory cue? How overlapped can spatiotemporal responses be

and still be perceptually unique? To answer these questions, carefully matched psychophysical and neurophysiological studies must be compared.

Studies in honeybee show that odor at low concentration elicits a restricted spatial response that becomes more distributed with increasing stimulus concentration (Sachse and Galizia 2003; Stopfer et al. 2003). Additionally, the amplitude of local field potential oscillations also increases with concentration suggesting greater oscillatory control upon spike timing (Stopfer et al. 2003). Likewise, behavioral studies in honeybee agree that odor discrimination (Wright, Lutmerding et al. 2005; Wright, Thomson et al. 2005) and discrimination learning (Pelz et al. 1997; Wright et al. 2002) are enhanced with increasing concentration. In addition, it has also been shown that odor quality (the perceived identity of the odor) might also change with concentration as well (Wright et al. 2002; Wright, Thomson et al. 2005). Thus, it is clear that there is a relationship between concentration and discrimination. However, the precise nature of the relationship between neural representations and behavioral measures of the ability to discriminate currently remains unclear. This is because behavioral and neurophysiological studies have not been experimentally matched.

Specifically, there is a need to correlate neurophysiological phenomena with an organism's olfactory capabilities in carefully matched psychophysical and neurophysiological experiments. *Manduca sexta* is a favorable model system for such a study for several reasons. Like the honeybee, this moth readily learns odor–food relationships (Daly and Smith 2000). This learned behavioral response forms the behavioral basis for psychophysical studies of olfactory function. In addition, this moth species is readily amenable to intracellular (Christensen and Hildebrand 1987; Christensen et al. 1996, 1998) and neural ensemble recordings (Christensen et al. 2000; Daly, Christensen et al. 2004; Daly, Wright et al. 2004; Lei et al. 2004). These neurophysiological methods have allowed detailed statistical analysis of spatiotemporal and stimulus-dependent population responses. These methods provide a basis for correlating psychophysical measures of the perceptual limits of olfactory function and the underlying neural representations that produced these limits. However, psychophysical methods for defining discrimination are currently undeveloped in this species.

Psychophysics is the study of the relationship between the properties of physical stimuli and their perceptual correlates. Psychophysical characterization of stimulus detection and discrimination thresholds are commonly measured behaviorally in both humans and nonhumans (Sarris 2006). In mammals, such as humans where can be more extensive than in insects and detailed accounts of sensory experience can be queried from the test subjects, there are several approaches for establishing measures of olfactory detection, discrimination, and identification (for review see Wise et al. 2000; Doty 2006). For example, using a triple-forced choice procedure in humans, it is possible to establish with reliability that 2 odors

can be discriminated (Hummel et al. 1997; Laska and Teubner 1999a, 1999b; Kobal et al. 2000). These methods have also been used in nonhuman primates as well (Laska and Freyer 1997). Of importance, this general method has established that odor discrimination is inversely related to the similarity of the comparison odors. Furthermore, as task demands increase by increasing the similarity between test odorant blend ratios, the latency of responses increase as well, this provides yet another indicator of discrimination (Wise and Cain 2000).

Unfortunately, such methods are not possible in our model system. Furthermore, there is no data characterizing discrimination thresholds as a function of concentration. Therefore, in the present study, we define the discrimination threshold as the concentration at which moths produce a statistically significant differential conditioned response (CR) to the reinforced (CS+) and non-reinforced (CS−) odors. Differential concentration-response functions, on the other hand, describe the rate at which the differential CR to the CS+ and CS− develops as a function of increasing concentration.

The primary goal of this report is to establish a psychophysical method that quantifies odor discrimination thresholds and differential concentration-response functions to the CS+ and CS− odors using a Pavlovian 2-odor discrimination assay in the moth *M. sexta* (Daly and Smith 2000; Daly, Chandra et al. 2001). Consistent with our prior studies of detection thresholds (Daly et al. 2007), it is expected that as concentration increases, the salience of the odors (i.e., perceived intensity) will increase but their perceived identity will remain constant. Thus as concentration increases, the differences in percentage of CRs elicited by the CS+ and CS− will increase. Assumed in the above hypothesis is that moths will always respond more to higher concentrations independent of the concentration at which they were conditioned.

A second goal of this report is to quantify the effect of stimulus salience on discrimination learning using the same discrimination assay. In this case, however, we will condition moths at and around identified discrimination thresholds. Here, we test the hypothesis that stimulus concentration affects the rate at which the animals learn (Rescorla and Wagner 1972). We also test how subsequent salience-dependent differences in learning, in turn, affect discrimination threshold measures. A salience-dependent negative impact on discrimination learning, we hypothesize, will in turn negatively impact measures of discrimination thresholds. Nevertheless, we expect that when tested at higher concentrations moths will always respond more at higher concentrations.

Materials and methods

Subjects

A total of 780 male and female *M. sexta* moths were used in the following study and represented in approximately equal

numbers in all experimental groups. Moths were shipped from Arizona Research Labs Division of Neurobiology, as stage 16–18 pupae. Upon arrival, individual pupae were put into brown paper bags. Pupae were then stored in a Percival incubator under a 16/8 reversed light/dark cycle at 25 °C and 75% relative humidity. Pupae were checked daily at the start of the dark cycle and bags containing newly eclosed adults were dated. Prior to use, adults were held 4–7 days posteclosion without food or water to increase motivation to feed.

Procedures

Moth preparation

Moths were placed in 1.27-cm inner diameter (ID) tubes so that the head and proboscis protruded. They were then restrained with a piece of a pipe cleaner and tape. The proboscis was extended and threaded into a 4 cm length of plastic tubing (1.6 mm ID; Tygon), which was then attached, with soft wax (Utility Wax; Kerr), to the tube that held the moth. A 0.125-mm diameter Teflon-coated silver wire (World Precision Instruments [WPI]) was placed into the right side of the head capsule bringing it into contact with the pharyngeal dilator muscle. A second silver wire served as reference electrode and was placed in the contralateral eye. Electrodes connected to leads, which in turn connected to a plug at the base of the restraining tube and allowed quick connection to a Plexiglas base. This base then integrated into the conditioning and testing stage. The stage contained leads for connection to and extracellular amplifier (DAM 50; WPI). The activity of the pharyngeal dilator and associated feeding muscles were monitored via electromyography (EMG) to quantify feeding behavior. EMG was monitored by the experimenter with an oscilloscope and a loudspeaker.

Stimulus delivery

The conditioning and testing stage consisted of an odor delivery system and an odor evacuation vent. Animals were placed in the center of the evacuation vent opening where a steady stream of airflow passed over the antennae. The velocity of this airflow was 0.2–0.3 m/s as measured with a hotwire anemometer. An odor cartridge was placed 10 cm upwind and aimed directly at the head of the moth. This distance ensured sufficient dispersion of the odorant such that the plume would interact with both antennae. This has been confirmed with liquid smoke tests (titanium–tetrachloride; Sigma-Aldrich) and is our standard practice (Daly and Smith 2000). For stimulation, air was supplied via a central air line. This air passed through a 500-cc Drierite cartridge to extract moisture and a custom 500-cc active charcoal filter. The charcoal filter was made from a Drierite cartridge in which the desiccant was replaced with fine charcoal granules (20–60 mesh; Sigma-Aldrich) to remove airborne olfactory contaminants. The output from the filter array passed through a flow meter (Cole-Parmer) set to 250 ml/min and into a 3-way valve (Lee Co.). Normally, air flows into one port of the valve then immediately out through a second port. When the valve is activated, the output is diverted to a third port, which is connected to the odor cartridge. The valve, as well as auditory cues for the experimenter to time the unconditioned stimulus (US) presentation, was controlled by a programmable logic chip (DL 05; Direct Logic), which allowed precise timing of the CS and US.

We used the same 6 odorants as in previous studies: racemic linalool (LOL), cis-nerolidol (NER), cis-3-hexenyl propionate (ZHP), methyl salicylate (MES), 2-hexanone (HEX), and 2-octanone (OCT). The first 4 odors were selected based on prior evidence suggesting that these are female-specific host-plant volatiles (Shields and Hildebrand 2000, 2001; Fraser et al. 2003). The last 2 odorants were selected based on their successful use in prior behavioral studies of olfactory function in this species (Daly and Smith 2000; Daly, Chandra et al. 2001; Daly, Durtschi et al. 2001; Daly et al. 2007). All odorants were at least 97% pure, details about them can be found in Table 1. Odorant cartridges were fashioned from glass tubing (6-mm ID) cut to a length of 7 cm. Nylon lure fittings (1/16 in ID; WPI) were inserted into either end of the glass tube. The internal volume of the cartridges was 1.5 ml after the fittings were inserted. Odorants were pipetted onto a piece of filter paper (No. 3 white; Whitman), which was cut into ~0.3 × 4 cm lengths and inserted into the cartridges. We established a 5-log step range of concentrations (0.5 ng/µl,

Table 1 Odorant name/(abbreviation), source, purity, density, vapor pressure (mmHg) at temperature (°C), initial dilution ratio (dilutions expressed µl mineral oil:µl odorant), and behaviorally identified detection threshold (see Daly et al. 2007)

Odorant/abbreviation	Source	Purity (%)	Density	Vapor pressure	Dilution ratio (µl)	Detection (µg/2 µl)
2-Hexanone	Sigma	98	0.81	10 at 39 °C	993.8:6.2	0.01
2-Octanone	Sigma	98	0.82	1 at 20 °C	993.9:6.1	0.01
Linalool	Sigma	97	0.861	0.17 at 25 °C	994.2:5.8	0.001
Methyl salicylate	Sigma	99+	1.18	1 at 54 °C	995.7:4.3	0.1
cis-Nerolidol	Fluka (Switzerland)	96	0.88	0.1 at 20 °C	994.3:5.7	10.0
cis-3-Hexenyl propionate	Sigma	97	0.89	11 at 25 °C	994.4:5.6	0.01

5 ng/μl, 50 ng/μl, 0.5 μg/μl, and 5 μg/μl) based on dilutions in mineral oil. When conditioning or testing with these dilutions, a 2-μl aliquot was placed on the filter paper. This volume produced a final dose range from 10 ng/2 μl to 10 μg/2 μl. Each dilution cartridge was used only once and replaced.

Response measures

The determination of a feeding response (whether conditioned or unconditioned) is based on an increase in feeding muscle activity and has been detailed elsewhere (Daly and Smith 2000). Briefly, as mentioned above, EMG electrodes were placed on the cibarial pump muscle, which is involved in sucking fluids from the proboscis into a cavity in the head capsule, the cibarium. The EMG activity of this and nearby associated feeding muscles are then amplified and output to a loudspeaker and oscilloscope. Spontaneously, these muscles are usually inactive. Upon presentation of a US, such as sucrose solution, the muscles reflexively activate producing strong (up to 6 mV) EMG spike trains that persist for several seconds. This activity is easily discerned on the loudspeaker and oscilloscope by the trainer/tester (for examples of EMG traces, see Daly and Smith 2000). The moth will, in many cases, also extend its proboscis, and this is used as an additional indicator of feeding activity.

Unlike the initial presentation of the US, upon initial presentation of the CS, there is typically no change in EMG activity and the proboscis does not extend. However, after one or just a few classical conditioning trials, where the CS is forward paired with the US, the CS begins to elicit activation of the feeding muscles and to a lesser degree extension of the proboscis. Importantly, this response occurs even in the absence of the US.

Once the US has been presented, however, there can be residual feeding activity that can span from trial to trial. Thus, during conditioning, a CR to the odorant is therefore only recorded if feeding muscle activity increases during the 3-s period from the start of the CS until presentation of the US. The criterion for establishing that a CR occurred as a result of CS presentation is therefore based on a subjective judgment by the trainer/tester that feeding muscle activity increased after the CS was presented. Although subjective, this method was found to produce the same results as direct measures of change in EMG spike rate but is far more practical (Daly and Smith 2000).

Data were recorded as 0 for no response and 1 for a CR. During conditioning, these data show the acquisition of a differential CR in response to the CS+ and CS− in a trial-by-trial manner. During test trials, where sucrose solution is not presented, the method was essentially the same but a 7-s period was used to assess the change in feeding activity. Seven seconds was used to allow for delayed CRs, which are common in moths.

Basic conditioning procedure

The Pavlovian-based differential conditioning protocols described here have been established in *M. sexta* and are

detailed elsewhere (Daly and Smith 2000). Briefly, to establish a differential CR between any 2 odorants that can be discriminated, 1 of the 2 odorants is reinforced (CS+) by forward-pairing it with sucrose solution, the US. For each CS+ trial, moths were presented with the CS+ for 4 s. Three seconds into CS+ delivery, the US, a 5-μl droplet of 0.75 M sucrose solution, is applied to the partially extended proboscis via a Gilmont syringe. US delivery is also 4 s in duration. This basic CS−US pairing is repeated 6 times. Pseudorandomly interspersed between CS+ conditioning trials, a second odorant is presented for 4 s but is nonreinforced (CS−). The delay between each of the 12 total trials was 6 min. Two pseudorandomized sequences were used in this study ("− + + − + − − + − + + −" and "+ − − + − + + − + − − +," where "+" are CS+ and "−" are CS− trials) on 2 subgroups of moths to ensure the CS+ both preceded and followed the CS− equally often.

Experiment 1: odorant discrimination thresholds with undiluted stimulus conditioning

The basic experimental design for Experiment 1 is displayed in Figure 1A. A total of 600 moths were used in this experiment. Four groups (n = 120 moths/group) were differentially conditioned with LOL and one of the following 4 odorants: NER, MES, ZHP, or HEX. In this case, LOL was used in all 4 odor pairs to have a common odor across all groups. An additional group (n = 120) was differentially
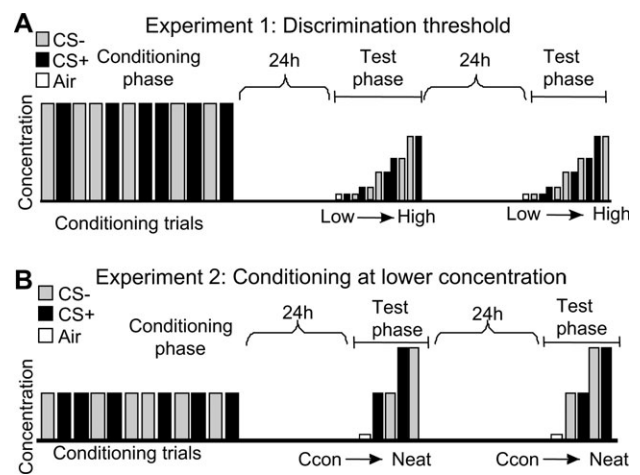

Figure 1   Schematic cartoon of Experiments 1 **(A)** and 2 **(B)**. In both experiments, moths were first differentially conditioned to the CS+ and CS− using 1 of 2 pseudorandomized patterns of presentation (see Materials and methods). At 24 and again at 48 h after conditioning, moths were tested with the same CS+ and CS− odors in random order but through a sequential increase in concentration. Note that there are 2 major differences between these experiments. First, in Experiment 1 moths are differentially conditioned with undiluted (or neat) odors. In Experiment 2, they were differentially conditioned with 1 of 3 different concentrations (only a single concentration is schematized) that were several log steps lower in concentration relative to Experiment 1. Second, the testing phases of the Experiment 1 used a 5-log step dilution series, whereas in Experiment 2, moths were tested with the concentration used for conditioning, then the undiluted odors.

conditioned to HEX and OCT. Groups were counterbalanced so that one-half of the moths in each group ($n = 60$) were conditioned with one odor as the CS+ and the second as the CS−. For the other half of the subjects, the CS+ and CS− odors were reversed. This reinforcement counterbalancing should produce symmetrical results if both odorants were equally salient.

Animals were conditioned using 3-μl aliquots of undiluted odorants. This minimized the likelihood that a lack of a CR during testing could be attributed to a salience-related lack of learning (Rescorla and Wagner 1972; Pelz et al. 1997; Wright and Smith 2004). That is, an undiluted odorant has the greatest intensity and hence should be the most salient.

Following differential conditioning, moths were returned to the incubator until testing, 24 and 48 h later. During the test phase, moths were first presented with a blank stimulus to assess baseline responsiveness to nonolfactory cues associated with the puffing of air. Next, moths were presented with the test odorants at the concentrations described above. Each concentration of the CS+ and CS− was presented once on each test day. Test stimuli were always delivered sequentially from low to high concentration in order to avoid extinction of the CR, particularly at the lower concentrations (Daly and Smith 2000). That is, every salient presentation of the CS+ (in particular) during the test phase represents an unreinforced trial. Repeated unreinforced presentation of a CS results in the eventual loss of the CR.

Experiment 2: effect of concentration on discrimination learning

To assess the effect of stimulus salience on discrimination learning, a separate set of differential conditioning experiments was performed using HEX and OCT. A schematic cartoon of the experimental design is displayed in Figure 1B. In this case, we used this pair to test whether differential conditioning above the detection threshold (previously identified at 0.01 μg/2 μl; Daly et al. 2007) but below the discrimination threshold (1.0 μg/2 μl as identified in Experiment 1) was sufficient to produce a differential CR, or whether at a minimum, these odors must be differently conditioned at or above the discrimination threshold. Furthermore, we can quantify whether differential conditioning above the discrimination threshold produced a greater overall differential CR. To test these hypotheses, 180 moths were differentially conditioned in 3 groups ($n = 60$/group). Moths were differentially conditioned and tested in counterbalanced subgroups ($n = 30$/subgroup) as previously described. Each of the groups was conditioned at one of 3 different concentrations: 0.1 μg/2 μl (one log step above the detection threshold but one log step below the discrimination threshold), 1 μg/2 μl (the discrimination threshold), and 10 μg/2 μl (one log step above the discrimination threshold). Discrimination threshold in this case is based on Experiment 1 results, whereas detection thresholds were defined elsewhere (Daly et al. 2007).

At 24 and 48 h postconditioning, each group was tested with both an odor blank and the CS+ and CS− odors, using the respective conditioning concentration for each group. The odor presentation sequence was randomized between individuals. Finally, moths were tested using undiluted concentrations of both the CS+ and CS−. This control tests whether moths had learned to differentiate between odors at the lower concentration but were simply unable to demonstrate evidence of that learning when tested at the low concentration.

Analysis

For both experiments, variation in CR was modeled using general linear modeling (GLM) in SAS. GLM analysis allows theoretical prespecification of variables and hierarchically partitions variance components for both categorical and continuous variables (Cohen J and Cohen P 1983).

In both experiments, variation in the percentage of moths eliciting a CR was modeled as a function of the following 6 main effects and their interactions: sex, the test day, odor identity, which was nested within odor pairs, concentration, and reinforcement (whether the stimulus was the CS+ or CS−). In Experiment 2, the concentration used to condition moths was also modeled. All possible 2- and 3-way interactions were modeled as well as select 4-way interactions and a $P < 0.01$ was used as the significance threshold to reduce experimentwise error rate. Post hoc means analysis was performed in SAS and implemented Tukey's honestly significant difference to maintain an overall $P < 0.05$. Curvilinear regression functions were calculated to predict the mean percent of moths eliciting a CR as a function of concentration. Although we modeled these functions using several different methods including linear, log-linear, and Weibull functions, the displayed results are based on third order polynomials as they tended to produce the best overall fit, based on R^2.

Results

Experiment 1: differential concentration-response functions with undiluted stimulus conditioning

The goal of this first experiment was to characterize discrimination thresholds and differential concentration-response functions in moths that were conditioned using the highest possible stimulus concentrations. Figure 2 displays the acquisition of the differential CR to the CS+ and CS− as a function of conditioning trials. In Figure 2A, results are collapsed across the reinforcement counterbalanced for the first 4 odor pairs because the counterbalance produced consistent results. Here, discrimination learning is evidenced by a systematic increase in mean CR elicited by the CS+ across trials with a concomitant decrease in the CR elicited by the CS− across trials. Figure 2B displays mean CR by trial for LOL and NER. In this case, note that moths did not acquire a differential response specifically when NER was the CS+.
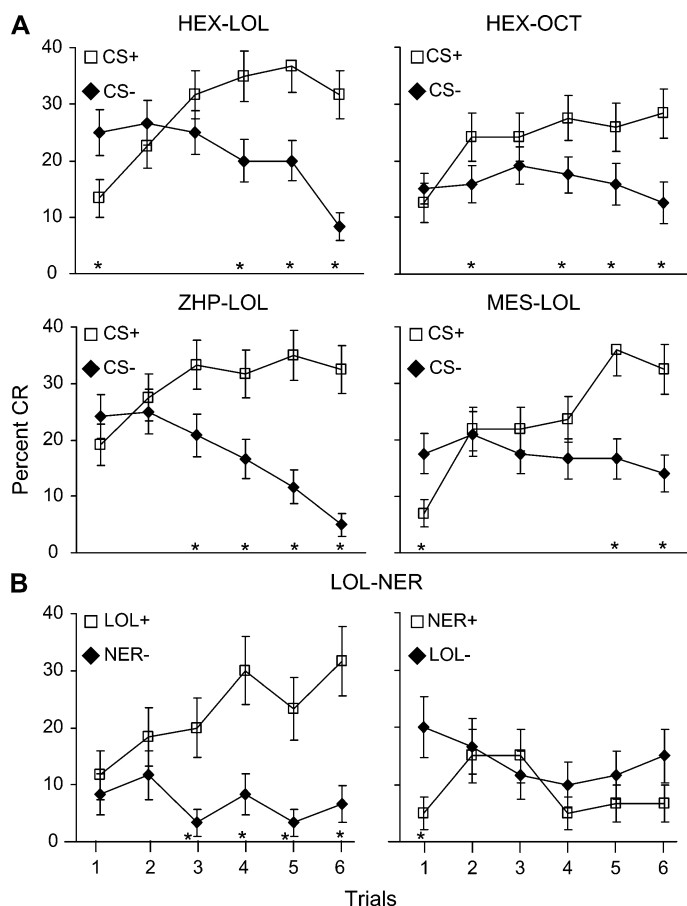
Figure 2 Acquisition of the differential CRs to the CS+ and CS− odors for Experiment 1; conditioning was with undiluted odors. Results presented as the mean percentage of moths eliciting a CR to the CS+ and CS− as a function of successive differential conditioning trials. **(A)** Mean acquisition for 4 different pairs of odors. Note in these cases, which odor was used as the CS+ or CS− did not affect the differential acquisition, and hence, the counterbalance has been collapsed. **(B)** Mean acquisition for NER and LOL broken down by the reinforcement counterbalance. Note here that, which odor of the pair had a profound impact on the acquisition of a differential response. In all panels, results of significant t-test comparisons between the %CR for the CS+ and CS− for each trial are inset along the x axis (*$P < 0.05$).
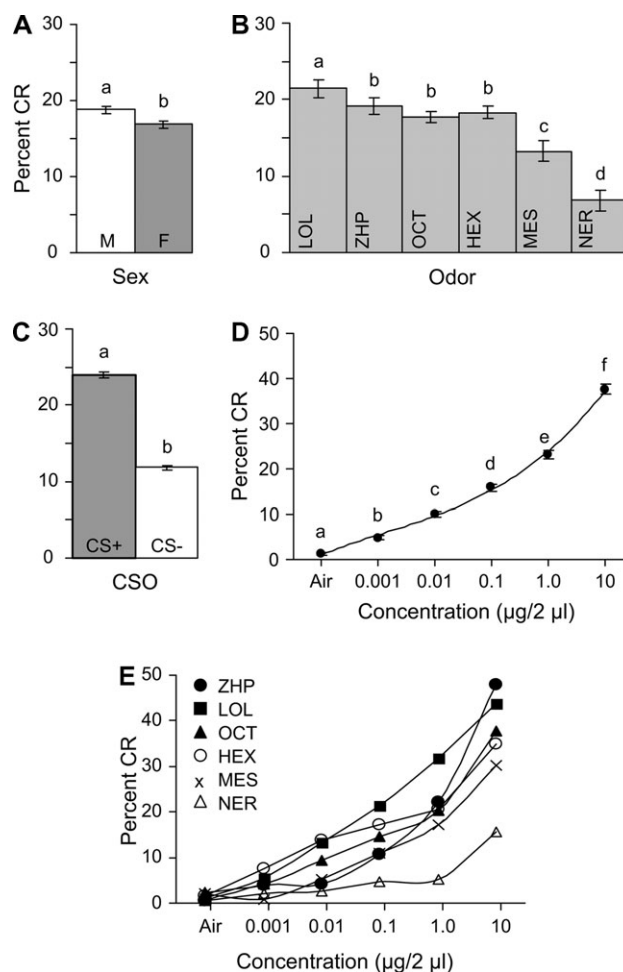

Figure 3 Mean percentage of moths eliciting a CR as a function of moth sex **(A)**, the test odorant **(B)**, reinforcement, which indicates whether the odorant was the CS+ or CS− **(C)**, stimulus concentration **(D)**, and the interaction of odor by concentration **(E)**. When appropriate results of post hoc analysis are indicated as letters; common letters above bars indicate nonsignificant differences between comparisons.

The overall statistical model explaining variation in the percentage of moths eliciting a CR for Experiment 1 during the test phase was significant ($P < 0.001$). Here, we find that males were 2% more likely to respond odor stimuli. This was a very small but significant effect ($P < 0.001$; Figure 3A). The main effect of odor (nested within odor pairs) was also significant ($P < 0.001$; Figure 1B) and indicates that different odorants produced different mean CRs.

The significant main effect of reinforcement ($P < 0.001$) indicates that when odorants were treated as the CS+ (i.e., reinforced), they elicited a significantly greater CR than when treated as the CS− (Figure 3C). The main effect of concentration was also significant ($P < 0.001$). As expected, as concentration increased the percentage of moths eliciting a CR increased as well (Figure 3D).

The above significant main effects, however, must be understood within the context of their significant interactions. For example, there was a significant interaction between odor and concentration ($P < 0.001$). As Figure 3E shows, this interaction indicates that each odor produced a unique concentration-response function. Thus, while all odors produce an increase in mean CR as a function of concentration, at which concentration the mean CR begins to increase and by how much is dependent on the specific odor stimulus.

The interaction of reinforcement by concentration, the key variable in this experiment, was significant ($P < 0.001$) and indicated that generally there was a systematic divergence in the mean CR elicited between the CS+ and CS− as a function of increasing stimulus concentration. This 2-way interaction was further dependent on the effect of odor pair, as indicated by a significant 3-way interaction of reinforcement by concentration by odor pair ($P < 0.001$). This 3-way interaction indicates that, although there was generally an increase in the mean

differential CR between the CS+ and CS− as a function of concentration, the particular combination of odorants used affected the amount of divergence that was observed. Finally, this 3-way interaction was dependent on which odor of the odor pair was reinforced as indicated by a significant 4-way interaction of reinforcement by concentration by odor pair by the reinforcement counterbalance ($P < 0.001$).

Although complex, this 4-way interaction can be readily understood by inspection of Figure 4. Figure 4A–E displays mean CR as a function of reinforcement by concentration; this is broken down in separate columns for each odor pair. For each respective odor pair, data are then further broken down (in rows i and ii) by the reinforcement counterbalance (i.e., which odorant of a pair was the CS+). The discrimination thresholds, defined here as lowest concentration at which a significant difference in the mean CR between the CS+ and CS− occurred (Tukey; $P < 0.05$), are denoted by an asterisk in each panel.

Figure 4A(i) shows that the percentage of moths eliciting a CR for LOL (CS+) increases as a function of concentration at a higher rate than for HEX (CS−). This systematic divergence between the CS+ and CS− as a function of concentration, we term the differential concentration-response function. The asterisk in Figure 4A(i) indicates that moths responded significantly more to the CS+ at 0.1 μg/2 μl, suggesting that moths can discriminate LOL and HEX at this concentration. This systematic increase in differential CR as a function of concentration is generally observed across Figure 4 (with one exception; see Figure 4B[ii]) and represents the significant concentration by reinforcement interaction. Note, however, that the concentration-response function for each odor pair across the top row (e.g.) in Figure 4 is unique. This odor pair–dependent difference in differential concentration-response functions represents the

significant 3-way interaction of reinforcement by concentration by odor pair.

Finally, when comparing the reinforcement counterbalance between Figure 4(i,ii) (i.e., upper vs. lower rows), it is clear that which odor of an odor pair was reinforced also affected the identified discrimination thresholds and the differential concentration-response functions in all but one case (see Figure 4E). Thus, counterbalancing which odor of an odor pair is reinforced will produce asymmetries in discrimination thresholds as well as differential concentration-response functions for most odor pairs. This asymmetry was most pronounced for the NER–LOL odor pair (Figure 4B[i,ii]). Here, moths produce a significant differential CR between NER and LOL at the second lowest concentration in the dilution series when LOL was the CS+ (Figure 4B[i]). On the other hand, when NER was the CS+, there was no evidence that moths respond to NER preferentially over LOL; in fact, quite the opposite occurred (Figure 2B[ii]). That is, moths responded more to LOL at 2 concentrations (inset X's). This is consistent with the acquisition data indicating that a differential CR was not acquired when NER was the CS+ (Figure 2B).

Experiment 2: effect of concentration on discrimination learning

We then asked how stimulus concentration affects discrimination learning and hence measures of discrimination thresholds by differentially conditioning moths using HEX and OCT at one-log step below, at, or one-log step above the identified discrimination thresholds as defined in Experiment 1. Figure 5A–C display the acquisition of differential CR's to the CS+ and CS− as a function of successive conditioning trials. Data are displayed separately for each concentration used to condition the 3 separate groups of moths. The first
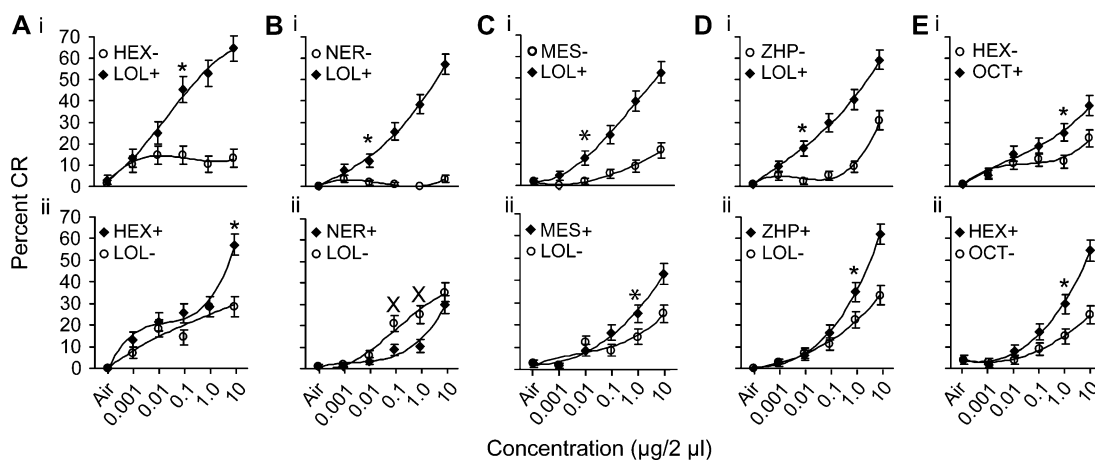

Figure 4 Mean percentage of moths eliciting a CR as a function of the 4-way interaction of reinforcement by concentration by odor pair by the reinforcement counterbalance. First, panels **(A–E)** break the reinforcement by concentration interaction down by odor pair. In columns i and ii, this is further broken down by the reinforcement counterbalance. Thus for example, A(i) and A(ii) display the differential responses to LOL as CS+ and HEX as CS−− (A[i]) and HEX as CS+ and LOL as CS− (A[ii]). Discrimination thresholds are identified by inset asterisk. Note in this case as in **(B–D)**, discrimination thresholds are dependent on which odor of an odor pair is reinforced; this is not the case with **(E)**.
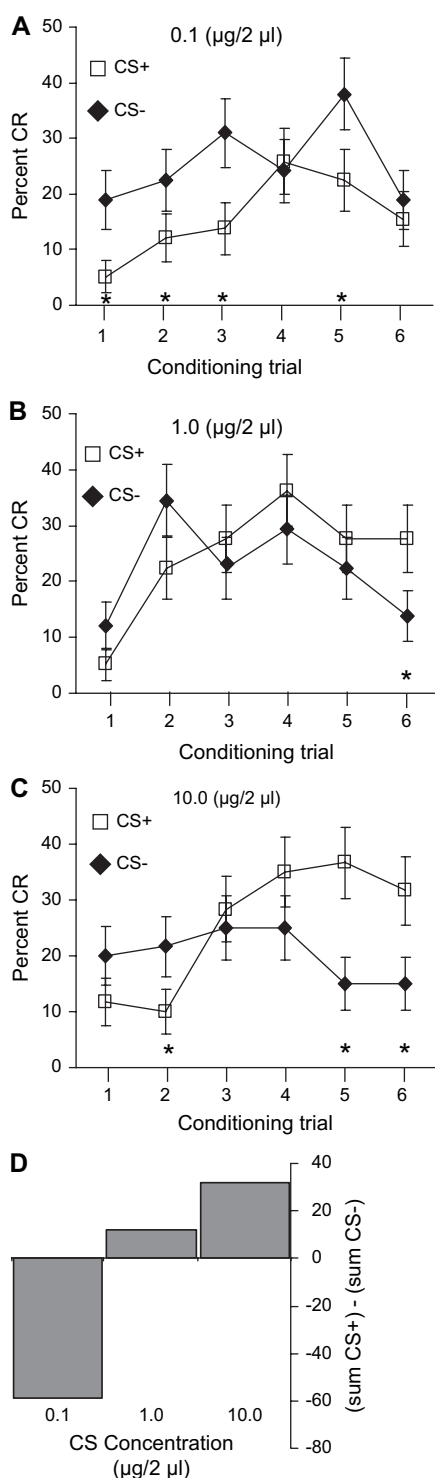
Figure 5 Discrimination learning as a function of stimulus concentration. **(A–C)** Differential percent CR for the CS+ and CS− odors as a function of successive conditioning trials at one-log step below **(A)** at **(B)** or one log step above **(C)** the discrimination threshold. In A–C, results of significant *t*-test comparisons between the %CR for the CS+ and CS− for each trial are inset along the *x* axis (*P < 0.05). **(D)** Bar graph summarizing the difference in CRs between the CS+ and CS− for the data shown in (A–C). Note that differential conditioning below the discrimination threshold produced a negative difference indicating that moths actually responded to the CS− more than the CS+ across conditioning trials.

and most obvious observation is that at the lowest concentration (Figure 5A), the CS+ never achieves a higher CR than the CS−. At the previously determined discrimination threshold (1.0 μg/2 μl; see Experiment 1 results) or above, the CR that is elicited by the CS− is initially greater but eventually CS+ comes to elicit a greater CR as a function of successive trials as seen in Figure 5B and C (see also Figure 2A). The degree of difference in CRs elicited by the CS+ and CS− thus provides an index of task difficulty. This can be quantified by subtracting the sum of all CR's elicited by the CS+ from the sum of all CR's elicited by the CS−. This is shown in Figure 5D and indicates that performance increases with concentration.

The overall statistical model explaining variation in CR for Experiment 2 posttests was significant ($P < 0.001$). The main effects of sex, day, and odor were not significant in this model. In the case of odor, the nonsignificant main effect indicates that both odorants were equally able to elicit a CR.

In the current experiment, we were not only interested in the 2-way interaction of reinforcement by test concentration, but also in the 3-way interaction of reinforcement by conditioning concentration by test concentration. This 3-way interaction indicates whether differential conditioning at different concentrations produced unique differential concentration-response functions. The main effects of reinforcement, conditioning, and test concentrations, all 2-way interactions and, most importantly, the 3-way interaction of reinforcement by conditioning concentration by test concentration were significant in this model ($P < 0.001$).

Figure 6A–C, displays the percentage of moths eliciting a CR as a function of test concentration and is broken down by conditioning concentration. In Figure 6A, moths were differentially conditioned with 0.1 μg/2 μl, which is one-log step below the identified discrimination threshold but one log step above previously identified detection thresholds for these odors. Note first, that moths responded more to the CS+ and CS− than to air and, furthermore, the percent CR increased as a function of concentration. Thus, given that naive moths respond to these odors around ~6–12%, Figure 6A indicates that they clearly learned that odorant predicted food. However, they did not differentially respond to the CS+ and CS− at either test concentration. This indicates that while moths learned that odor predicts food, they were unable to discriminate between odors.

In contrast, Figure 6B establishes that moths conditioned at the identified discrimination threshold (1.0 μg/2 μl) differentially responded to the CS+ and CS−. This difference was not significant at the conditioning concentration, but was clearly significant at the higher concentration. This indicates that moths learned to differentially respond to odorants when conditioned at the discrimination threshold. Finally, as expected, Figure 6C shows that when moths are differentially conditioned at one log step above the discrimination threshold, they encounter no difficulty in establishing a significant differential response to the CS+ and CS− at both the conditioning and undiluted concentrations.
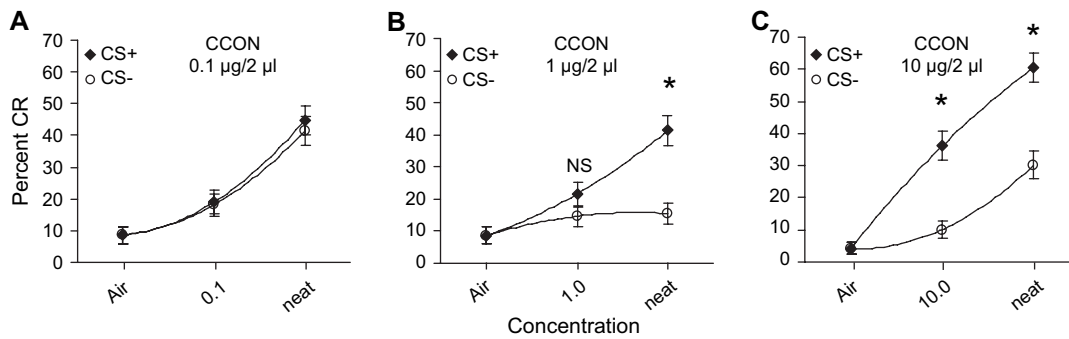
Figure 6 Mean percentage of moths eliciting a CR as a function of test concentrations and whether the odor was reinforced (CS+) or not reinforced (CS−). **(A)** Moths that were differentially conditioned at 0.1 μg/2 μl. **(B)** Moths that were differentially conditioned at 1.0 μg/2 μl. **(C)** Moths that were differentially conditioned at 10.0 μg/2 μl. Inset asterisk indicates significant difference in the percent of moths eliciting a CR between CS+ and CS− in post hoc analysis ($P < 0.05$).

Discussion

The problem of identifying psychophysical thresholds for discrimination is difficult in invertebrates because of the relatively limited behavioral paradigms available. Yet, establishing these thresholds are essential, if we are to understand what spatiotemporal aspects of neural representations are necessary and sufficient for olfactory percepts to emerge. We have shown in a previous report that detection thresholds and concentration-response functions can be generated using a Pavlovian paradigm (Daly et al. 2007). In the present study, we provide differential concentration-response functions for pairs of odors. These functions describe the rate at which these pairs of odors become perceptually distinct as concentrations increase and allow us to identify a discrimination threshold. In comparison of this and our previous report, which quantified odor detection thresholds, we can conclude that discrimination requires a higher level of sensory input than detection. We make this conclusion based on the observation that moths detect these odors at concentrations one or more orders of magnitude below that which is necessary for discrimination (see Table 1).

Furthermore, when moths are conditioned to discriminate using concentrations, which are above the detection threshold but below the identified discrimination threshold, they are simply unable to differentially respond to the reinforced odor, independent of the test concentration. Moths learn to respond to both the CS+ and CS− odors in this case, indicating that they detect both odors at these concentrations, but that there is insufficient odor information for them to establish unique representations for the CS+ and CS−. This strongly supports the conclusion that detection and discrimination require different levels of information (Wright and Smith 2004). It is possible that additional conditioning trials could alter this conclusion, but this seems unlikely given that we have consistently observed that moths acquire a CR in as few as 1 or 2 trials (Daly and Smith 2000; Daly, Chandra et al. 2001; Daly, Durtschi et al. 2001).

In Figure 6B,C, when moths were tested with the undiluted CS+ and CS−, both groups differentially responded about equally. This occurred in spite of the fact that the group represented in Figure 6C was differentially conditioned with more salient (higher concentration) stimuli. One might expect that there should be a greater differential response in this case. On the other hand, these results suggest to us that discrimination ability has reached an asymptote for these 2 closely related odors. That is, moths can perform no better at discriminating between these odors, independent of increases in concentration. Indeed, previous comparison of 6 versus 8 carbon alcohols yielded the same results when differentially conditioned and tested with undiluted stimuli (Daly, Chandra et al. 2001).

We also find that defining discrimination thresholds is more complex than simply assessing the concentration at which animals produce a significant differential CR to the reinforced and nonreinforced odors. Not surprisingly, this is because equal dilutions, based on the density of the individual odors, do not equate to equivalent saliences at the level of sensory perception. This occurs for 2 mutually exclusive reasons. First, density does not equate to volatility. In this case, density was used for comparison with other ongoing studies. Calibrating based on vapor pressure would provide more equivalent concentrations impacting the antenna. The second reason is that independent of volatility, olfactory systems are not equally sensitive to all odors; this too is borne out in our detection threshold studies as well.

When differences in the detection thresholds (see Table 1) are compared with the asymmetries in discrimination thresholds, we observe that the differences in detection thresholds predict asymmetries in discrimination thresholds. Specifically, we find that when odors with low detection thresholds, such as LOL, are the CS+ and odors with higher detection thresholds are the CS−, the result will always be a lower discrimination threshold. By accounting for differences in odor volatility and, more importantly, the overall sensitivity of the animals using psychophysical methods, we expect that

asymmetries in the reinforcement counterbalance could be greatly reduced.

Our first set of experiments was designed to minimize the effect stimulus salience has on olfactory learning by conditioning the moths at the highest concentrations. Previous studies of discrimination learning have recognized that as the concentration of a stimulus is lowered, its salience is lowered as well (Pelz et al. 1997; Wright and Smith 2004). Stimulus salience is a key factor that directly influences the rate of learning (Rescorla and Wagner 1972). Hence, as salience is lowered learning is slowed. We show that impaired learning in turn biases discrimination threshold measures to be higher. This is confirmed by comparison of discrimination thresholds for HEX and OCT in Experiments 1 and 2. Experimentally, this bias has typically been compensated for by adding more learning trials. An alternative approach that we applied here is to simply condition animals at high stimulus concentrations and test with a panel of increasing concentration. This ensures that the overall learning is roughly comparable across groups. In this case, potential differences in salience can result from differences in antennal sensitivity. Disruption of learning can also occur because of an innate biological meaning of an odor, such as in the cases of pheromones and kairomones (see Daly, Durtschi et al. 2001; De Moraes et al. 2001).

Our method assumes that there is a perceptual invariance of odor object quality across concentration. That is, across the range of concentrations we used, we assume that if the animals can identify odor X they invariably perceive it as odor X independent of concentration. Of course, it is not possible to unambiguously demonstrate perceptual invariance in nonhumans and there are examples in the human psychophysical literature indicating that identity can change (Gross-Isseroff and Lancet 1988). However, our results seem to indicate consistently that although these animals are sensitive to changes in intensity of a stimulus, so long as there is sufficient concentration to identify an odor, they perceive the identity as unchanging. Thus, increasing concentration increases the salience of a stimulus and hence invariably produces an increase in CR (or differential CR), independent of the concentration used to condition the animals. In fact, we consistently observed that even when conditioned at lower concentrations, there was always an increase in the number of moths eliciting a CR when tested at higher concentrations.

Studies of olfactory receptor neurons indicate that as concentration increases, the spatial distribution of odor representations become both more distributed and more overlapped simply because as concentration increases the specificity of olfactory receptor neurons decreases (Malnic et al. 1999). One might predict that this would produce an increase in the overlap between representations within primary olfactory networks, such as the AL or OB. Yet, at the level of processing and output, less specific input yields spatiotemporal responses that become broader but may be more distinct from responses to other odorants (Sachse and Galizia 2003; Stopfer et al. 2003). This general pattern of increasing uniqueness of odorant representations in primary olfactory networks is consistent with the behavioral data described here and elsewhere (Pelz et al. 1997; Wright and Smith 2004; Wright, Lutmerding et al. 2005). What these correlated psychophysical and neurophysiological results suggest is that both broad tuning of olfactory receptor neurons and sufficient concentration are necessary prerequisites for olfactory systems to perform typical odor discrimination tasks.

This conclusion appears at odds with some data suggesting that olfactory systems evolve specific olfactory receptors for specific odorant ligands, such as host plant odors and pheromones. For example *M. sexta* ALs contain a female-specific glomerulus that is both enlarged and particularly sensitive to racemic (+/−) LOL at concentrations comparable to the lowest concentrations used herein (Reisenman et al. 2004, 2005). However, our behavioral studies show males are quite sensitive to this odor too. Indeed, post hoc comparison of males versus females indicates that, whereas females may be slightly more responsive at the very lowest concentration, males did as well at all subsequent concentrations (sex comparisons not shown). The presence of adaptive specializations like male- and female-specific glomeruli cannot be denied and these need to be studied within the context of the behaviors they elicit in their natural ecologies. However, the number of glomeruli that represent adaptive specializations for the processing of pheromone- and kairomone-type odorants seem to represent only a small minority of the total. Furthermore, the generalist foraging strategy of hawk moths, such as *M. sexta*, which have been observed to feed on hundreds of flower species (Fleming 1970; Haber and Frankie 1989), suggests a relatively broad olfactory tuning and necessitates a psychophysical approach that is capable of quantifying behaviorally the perceptual limits of olfactory systems in a more general sense.

Supplementary material

Supplementary material can be found at http://www.chemse. oxfordjournals.org.

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