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The generalization of an olfactory-based conditioned response reveals unique but overlapping odour representations in the moth *Manduca sexta*

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Summary

Most highly derived olfactory systems, such as the insect antennal lobe, discriminate among a wide array of monomolecular odourants and blends of odourants. Given the relatively limited number of neurons used to code these odours, this ability implies that neural representations for odours overlap in a cross-fiber coding scheme. Here we use the generalization of a conditioned feeding response in the sphinx moth, *Manduca sexta*, to quantify three geometry-based dimensions of odour space in which monomolecular odours may be assessed. In a series of experiments we show that generalization of a conditioned response from one monomolecular odour to another is a function of differences in length and shape of the carbon chain as well as the functional group on the molecule. When moths were conditioned to 2-hexanone or 1-decanol and tested with a number of alcohols and ketones, we found that the generalization of the conditioned response decreased as a function of the chain length and functional group. In contrast, when conditioned to 1-hexanol, moths failed to distinguish alcohols from ketones of the same chain length. In all of these cases, chain length did not interact with functional group, thus indicating the independence of these dimensions. Differential conditioning of alcohols and of alcohols and ketones revealed interaction of excitatory and inhibitory generalization gradients within an odour 'dimension'. When odourants were sufficiently distinct, the peak of the generalization gradient was shifted away from the conditioning odour and in an opposite direction from the unreinforced odour. Altogether, these data substantiate the claim that these molecular characteristics are relevant coding dimensions in the moth olfactory system. These data are consistent with a cross-fiber coding scheme in which odours are coded by spatio–temporally overlapping sets of neurons, both in the periphery and in the antennal lobes.

Key words: Olfaction, odour coding, odour space, conditioning, discrimination learning, stimulus generalization, *Manduca sexta*.

Introduction

Studies of several species of moths have begun to reveal the physiological basis that underlies behavioral responses of males to female sex pheromones (Hildebrand and Shepherd, 1997). Once detected by males, these odours, 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). Specialized processing of these pheromonal components begins with sensory transduction and is continued at the first layer of synaptic interaction in the antennal lobes (AL; Christensen and Hildebrand, 1987; Waldrop et al., 1987; Christensen et al., 1998). In this case, early processing is a reasonably narrowly tuned subsystem within the male moth's olfactory system, which presumably evolved because of the reliability of the pheromonal signal over evolutionary time (Smith, 1996).

Moths like *Manduca sexta*, also respond to an array of non-pheromonal plant odours, and it is becoming increasingly clear that a great many plant odours are processed by specific subsets of sensory receptors (Anderson et al., 1995; Jonsson and Anderson, 1999; Shields and Hildebrand, 2001) and central neural pathways (Anton and Hansson, 1994; Anton and Hansson, 1995). Some of these odours are capable of eliciting stereotypic, or innate, behavioral responses (Anderson et al., 1993). For instance, odourants emitted from larval frass (Anderson et al., 1993) have been shown to influence a female moth's oviposition behavior. In cases such as these, there appear to be appropriate specializations of the olfactory system that are analogous, at least in terms of tuning properties, to the sex pheromone subsystem.

M. sexta also regularly feeds on floral nectar. It is not surprising, therefore, that recent studies of this and other moth species have revealed a capacity to learn the relationships between odour and sucrose reinforcement (Hartlieb, 1996; Fan et al., 1997; Daly and Smith, 2000), similar to other nectar foraging insects such as the honeybee (Menzel and Bitterman, 1983). Detailed investigation into the mechanisms that underlie olfactory-based learning have revealed contributions from both non-associative and associative processes (Hartlieb et al.,

1999; Daly and Smith, 2000). Simply feeding sucrose solution (an unconditioned stimulus, US), regardless of how it is paired with odour (a conditioning stimulus, CS), can increase the rate of background feeding-related activity (Daly and Smith, 2000), which is indicative of sensitization. More dramatic increases in feeding responses, which are cued by odour and are maintained for at least 24 h, require contiguous forward pairing of odour and sucrose (Menzel and Bitterman, 1983; Hartlieb et al., 1999; Daly and Smith, 2000). Furthermore, moths and bees can be easily conditioned to discriminate an odour that is paired with sucrose reinforcement from a second odour that is not paired with reinforcement. The specificity of the CS/US relationship in the forward pairing of a single odour and in the differential conditioning of two odours are indicative of associative processes (Daly and Smith, 2000).

Plant odours that must be learned cannot be coded by way of a labeled-line system (Smith, 1996). This is because of the potentially vast number of odours that could be relevant to a generalist feeder like the honeybee or sphinx moth. These animals must forage in environments which lack predictability on an evolutionary time scale, but can provide consistent cues that predict the presence of food within the animal's lifespan (Cunningham et al., 1998). Recognition of these predictive odour cues increases an animal's fitness.

It has been shown that any given monomolecular odour or blend is processed in defined regions of the vertebrate olfactory bulb (OB; Mori et al., 1992; Katoh et al., 1993) as well as in the insect antennal lobe (AL; Galizia et al., 1999; Galizia et al., 2000). In addition there is an array of unique temporal components to these odour representations that can be observed in the olfactory systems of both vertebrates (e.g. Kashwadani et al., 1999) and invertebrates (Stopfer et al., 1997; Sandeman and Sandeman, 1998; Gelperin, 1999; Teyke and Gelperin, 1999; Laurent, 1999) in response to odour presentation. These studies suggest that there is an explicit spatio–temporal cross-fiber code for each odour.

Currently, there is a need for a more thorough investigation of how subtle and systematic variations in the structure of odour molecules can influence behavior. Systematic exploration of monomolecular odours, such as incrementally increasing chain length within a functional group, allows us investigate how the most basic changes in the structure of a molecule can affect odour perception and hence can inform us about how odours are coded in the brain. This behavioral information should, furthermore, correspond to information from physiological investigations of neural representations of odour. Odours that animals have greater difficulty discriminating between should show greater overlap in neural representations and, in general, these odours should be geometrically similar as well. In the rabbit OB, for instance, it has been shown that mitral/tufted cells have specific tuning properties that are sensitive to subtle variation in functional group, hydrocarbon chain length and functional group position on the molecule (Imamura et al., 1992; Katoh et al., 1993).

However, while these studies suggest that different odours elicit responses from unique yet overlapping ensembles of neurons, the question of whether these variations amount to salient differences in odour perception must be explored within a behavioral context. Here we extend work in the honeybee (Hosler et al., 2000; Smith and Menzel, 1989a; Smith and Menzel, 1989b; Smith, 1993) and sphinx moth (Daly and Smith, 2000) to incorporate analysis of odours that vary in the dimensions described above. To that end, we applied two basic methodologies to assess potential odour-coding schemes. First, we conditioned a feeding response to a single odour and assessed the degree to which that conditioning generalizes to other odours (Smith and Menzel, 1989b; Smith, 1993; Daly and Smith, 2001). Second, we used differential conditioning of two odours of varying relatedness, again using the generalized response to assess the degree to which odour representations overlap. Both of these approaches have been used to investigate the dimensionality of other stimulus domains such as vision (Hanson, 1959; Mood et al., 1991) and audition (Weinberger, 1998). Specifically we explored how systematic variation of carbon chain length, carbon chain shape and functional group influences the strength of a previously learned response to a given odour.

Materials and methods

Subjects

Male and female *Manduca sexta* (Johansson) were obtained at or near stage 18 of pupal development from Arizona Research Laboratories, Division of Neurobiology, *via* overnight delivery. Rearing conditions have been described (Bell and Joachim, 1976). Upon arrival, pupae were sexed and isolated in brown paper bags where they remained undisturbed until used. The paper bags were placed in environmental chambers that held the temperature at 28 °C, at 80 % relative humidity, under a 16 h:8 h L:D cycle.

Bags were inspected once daily prior to the start of the dark cycle. Eclosion dates were recorded on bags in which newly emerged pupae were found. Age at the initiation of training was between 4–8 days post-eclosion. We have found that holding moths, without food or water, increases motivation to feed without hindering performance (Daly and Smith, 2000). Once subjects were of the proper age they were randomly assigned to one experimental group and used only once.

Preparation

The conditioning methodology described below has been successfully implemented in prior studies (Daly and Smith, 2000; Daly et al., 2001) and was adapted from the proboscis extension response (PER) conditioning protocol in the honeybee (Menzel and Bitterman, 1983). Briefly, subjects were restrained in individual plastic tubes with the proboscis partially extended through a piece of flexible surgical tubing, leaving the distal end of the proboscis exposed. Teflon-coated fine silver wire electrodes were placed just under the surface of the head capsule, between the compound eye and the sagittal midline, bringing it into contact with the pharyngeal dilator

muscle (Eaton, 1971). A second electrode was placed in the contralateral compound eye for reference. The electrodes were connected to an A-M Systems model 1700 differential AC amplifier; the output of this amplifier led to an oscilloscope and to an audio output device.

Odour cartridges were made from 1 ml tuberculin glass syringes. Inserted into these syringes was a strip of filter paper upon which 3 μl of odourant was placed. Odour cartridges were dedicated to a single odour to avoid cross contamination. All odour cartridges, whether used in conditioning or test trials (described below), were prepared with 3 μl undiluted dosages. None of the odourants were known pheromone components or plant volatiles that elicit stereotypic behavioural responses in *M. sexta*.

The odour cartridge was positioned at the front of the training stage and connected to an aquarium pump air supply. A computer controlled shunt diverted airflow to the odour cartridge at a rate of 7.5 ml s^{-1}. At the back of the training stage an exhaust port evacuated spent odourant from the training area. For each trial, subjects were placed in the center of the stage such that the odour cartridge was aimed directly at the subject's head from a distance of approximately 9 cm. This was an adequate distance to produce a dispersion field wide enough to cover both antennae (confirmed with titanium tetrachloride tests). When the shunt was opened, odourant was gently blown into the air-stream produced by the exhaust and then over the subject's head and antennae. The time for the odour to travel from the odour cartridge to the antennae has been estimated from antennal lobe recordings, using the same configuration, to be approximately 200 ms (K. C. Daly and others, personal observation). During the conditioning phase of each experiment the forward-paired conditioning odour (CS) was followed by presentation of a 0.75 mol l^{-1} sucrose solution (US) to the proboscis. The odour and sucrose were presented for a total of 4 s each. Sucrose was always presented 3 s after odour onset, producing a 1 s CS/US overlap.

Response measurements

Behavioural response measurements were based primarily on changes in the rate of electromyographic (EMG) activity from the cibarial pump muscle, and this procedure has been previously described (Daly and Smith, 2000). Subjects were scored on the basis of a detected change in feeding behaviour upon presentation of the CS. This score was based on three indicators: sound from the audio output, oscilloscope output and extension of the distal end of the proboscis. These are highly redundant measurements. However, use of all three indicators allowed the observer to pay close attention to the timing of CS and US while simultaneously monitoring behaviour. During conditioning trials, if the experimenter observed an increase in activity, as indicated by any combination of these indicators within 3 s subsequent to CS onset and prior to US delivery, a response was recorded for that trial. During test trials a 4 s period (the total time of odour presentation) was used.

Table 1. *List of odours used, their source, purity and molecular mass*

Odour	Source	Molecular mass (kDa)	Purity (%)
Cyclohexanone	1	98.15	99.80
1-Hexanol	1	102.18	99.00
1-Heptanol	2	116.20	99.00
1-Octanol	2	130.20	99.00
1-Nonanol	2	144.30	98.00
1-Decanol	1	158.29	99.00
2-Hexanone	1	100.16	98.00
2-Heptanone	1	114.19	98.00
2-Octanone	1	128.22	98.00
2-Decanone	1	156.27	98.00

1, Aldrich Chemical Co., Inc.
2, Sigma Chemical Co.

(1) Effect of functional group, carbon chain length and shape on generalization

The goal of the first experiment was to establish the degree to which a conditioned response (CR) generalized from a conditioning odour to odourants that differed in terms of carbon chain length, shape and functional group. A total of 240 subjects were conditioned in three groups. The first group (N=80) received six conditioning trials during which 1-hexanol was forward-paired with sucrose solution (see Table 1 for odour sources and purity). For the second group (N=80), 2-hexanone was forward-paired with sucrose solution. Each conditioning trial was separated by a 6 min inter-trial interval.

In the first two groups, any decrease in generalization of the CR that occurs as a function of chain length will covary with odour volatility. Odours with greater chain lengths have higher molecular mass and hence are less volatile. This potentially confounding factor was therefore assessed in the third group, where we conditioned moths (N=80) to 1-decanol, the odour with the highest molecular mass (see Table 1), and measured generalization in post-tests to 8-, 7- and 6-carbon alcohols and ketones, and to cyclohexanone. If odour volatilization influences the gradient of generalization we would expect the absolute value of the slope of the chain length variable to be appreciably shallower in the third group.

Typically, during and immediately following conditioning, moths display considerable spontaneous feeding activity, which probably occurs because of either residual sucrose on the proboscis or sensitization (Daly and Smith, 2000). A 2-hour holding period was therefore used to ensure that all spontaneous feeding activity ceased prior to testing. In the test phase, each moth was tested with the conditioning odour and seven other odours, presented separately, without reinforcement and in a randomized sequence across subjects. In the first two groups, the test odours were three alcohols of increasing carbon chain length: 1-heptanol, 1-octanol and 1-decanol, and three ketones of increasing chain length, 2-heptanone 2-octanone and 2-decanone. In the third group we

again used three alcohols and three ketones but this time they were of decreasing chain length from 8 carbons to 6. We also tested responses to cyclohexanone in all three groups to evaluate the effect of the shape of the carbon chain. In this latter case, cyclohexanone is identical in composition to 2-hexanone, but the carbon chain forms a ring. Odour presentation was randomized across subjects to control for extinction and sequence effects. Each trial was scored according to whether the observer detected an increase in feeding-related behavior after odour onset (see above).

(2) Differential conditioning

Another method that has been used to assess the dimensionality of sensory representations is through differential conditioning of two odours, which might produce inhibitory and excitatory generalization gradients (see Discussion). If these gradients exist along the same perceptual dimension and overlap, they should summate to produce asymmetric gradients around the excitatory stimulus (Spence, 1937). In the present experiment each subject (N=130) received a total of twelve conditioning trials, six with each of two odourants (R and N) presented in a pseudo-randomized sequence. Odour R was reinforced with sucrose while odour N was presented without reinforcement. Moths were conditioned in subgroups of equal numbers using one of the following two patterns of pseudo-random presentation: RNNRNRRNRNNR and NRRNRNNRNRRN. The second sequence is simply a counterbalanced control for the sequence of odour presentation. As in experiment 1, a 6 min interval was maintained between trials. However, in contrast to experiment 1, because reinforced trials were interspersed with non-reinforced trials with a second odour, the interval between reinforced trials ranged between 6 and 18 min. Observational data were collected for all conditioning and test trials as described above.

Moths were subdivided into three groups and differentially conditioned to different pairs of odours. The first group (N=40) was differentially conditioned to 1-heptanol (R) and 1-hexanol (N). 2 h after training each moth was tested with the 6-, 7-, 8- and 10-carbon alcohols. The second group of moths (N=50) was differentially conditioned to 1-octanol (R) and 1-hexanol (N). Moths in this group were tested with 6-, 7-, 8-, 9- and 10-carbon alcohols. The third group (N=60) was conditioned to 1-hexanol (R) and 2-octanone (N) and post-tested to 6-, 7- and 8-carbon alcohols and ketones. Differences in the number of moths per group reflect differences in the number of odours used in post-tests (10 moths per odour used).

Statistical 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. Data were recorded as 0 for no response and 1 for a response. These data were used to create acquisition curves, which show the probability of subjects displaying a CR by trial for each odour used.

During test trials a 4 s period was used to assess the change in feeding activity and responses were recorded in the same manner as the acquisition data. Here, general linear modeling (GLM) analysis was used to analyze variation in responses from experiment 1 and from the third group in experiment 2. GLM analysis allows for theoretical pre-specification of variables and hierarchically partitions variance components for both categorical and continuous variables (Cohen and Cohen, 1983). Furthermore, it not only provides information about the magnitude of each significant variance component but also provides slope information (SAS Institute, 1989). This allowed us to investigate, for instance, whether odour volatility affects the slope of the generalization gradient by direct comparison of slope estimates from different groups.

Four variables and one interaction term were created to explain the variation in response probabilities. The first variable was sex, which in our preliminary analysis was insignificant across all groups. In addition we tested all possible 2-way interactions with sex and the other variables described below and again found no significant effects. Thus sex was omitted from the final models. The second variable was chain length, which was treated as a continuous variable that ranged between 6 and 10, reflecting the number of carbon units in the carbon chain of each odour. The third variable was functional group, which was also treated as a continuous variable, specifically coded as 0 and 1. The parameter estimate calculated by the GLM therefore provides an unbiased estimate of the magnitude and direction of this main effect. The final variable we created was molecule shape. This variable also contained only two levels, round and straight, to reflect the shape of the carbon side chain. However, because we used only one cyclic ketone (cyclohexanone), we felt that it would be conservative to treat it as categorical. The only interaction term created for these models was carbon chain length by functional group.

The experimental design of the first two groups in experiment 2 was relatively simple. In these two groups we did not vary functional group or shape; all odours were alcohols. Our interest here was to simply to make comparisons between mean response probabilities P to show asymmetries on either side of the generalization gradients. This was most easily accomplished by making one-tailed t-test comparisons between means. Values are means ± S.E.M.; significance levels for all t-tests were 0.05.

Results

Fig. 1 displays acquisition of the CR for the conditioning odours used in experiments 1 and 2. The initial probability of a spontaneous feeding response for each odour was relatively low (0.07, 0.11 and 0.12 for 2-hexanone, 1-hexanol and 1-decanol, respectively). There was a general trend across all three odourants to reach a peak probability P of approximately 0.50 by trials 3–5, which was followed by a

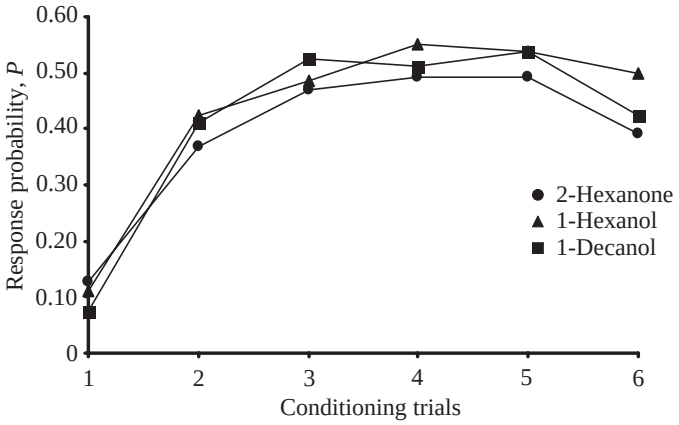

Fig. 1. Acquisition curves displayed as the probability *P* of moths responding to odour presentation, prior to sucrose presentation, for the three odours used in the conditioning phase of experiment 1: 1-hexanol (*N*=80) 2-hexanone (*N*=80) 1-decanol (*N*=80). Note the clear and consistent pattern across all three odourants of acquisition that peaks between trials 3 and 5 then begins to decrease on the sixth trial.

slight decrease after the fifth trial. Both of these trends are consistent with our prior work, which demonstrates that this acquisition is attributable to associative processes (Daly and Smith, 2000).

Effect of functional group, carbon chain length and shape on generalization

Fig. 2A shows the mean CR probability for the conditioning odour (1-hexanol) and for 7 test odours. Statistical analysis (Table 2A) confirms that as the chain length of the test odour increased there was a significant decrease in response probability *P*. The regression equation in Table 2A indicates that with each incremental increase in chain length there is a corresponding 9% decrease in probability of a feeding response. There is also a significant effect of chain shape. Fig. 2A indicates that cyclohexanone produced a smaller response than 1-hexanol, which suggests that the shape of the carbon chain has a greater effect on generalization than functional group in this case. Table 2A also indicates that there was no effect of functional group. That is, after accounting for the influence of chain length and shape, we found that for any given chain length, the response generalized equally to alcohols and ketones.

Fig. 2B shows mean response probabilities for the conditioning odour (2-hexanone) and test odours. As before, there was a significant effect of chain length (Table 2B), which produced a 4% decrease in response probability with each incremental increase in chain length difference between the conditioning odour and test odour. Table 2B also indicates that there was a significant effect of functional group. Because we treated functional group as a continuous variable coded as 0 and 1, the parameter estimate, in this case –0.17 (see Table 2B), provides an unbiased estimate of the effect of switching from ketones to alcohols. In this instance, for any given chain length, the probability of a feeding response decreased by 17% when switching from a ketone to an alcohol. Finally, there was a significant effect of carbon chain shape. Comparison of means (Fig. 2B) indicates that cyclohexanone again had a lower probability of eliciting a response than other 6-carbon molecules, supporting the results from group 1.

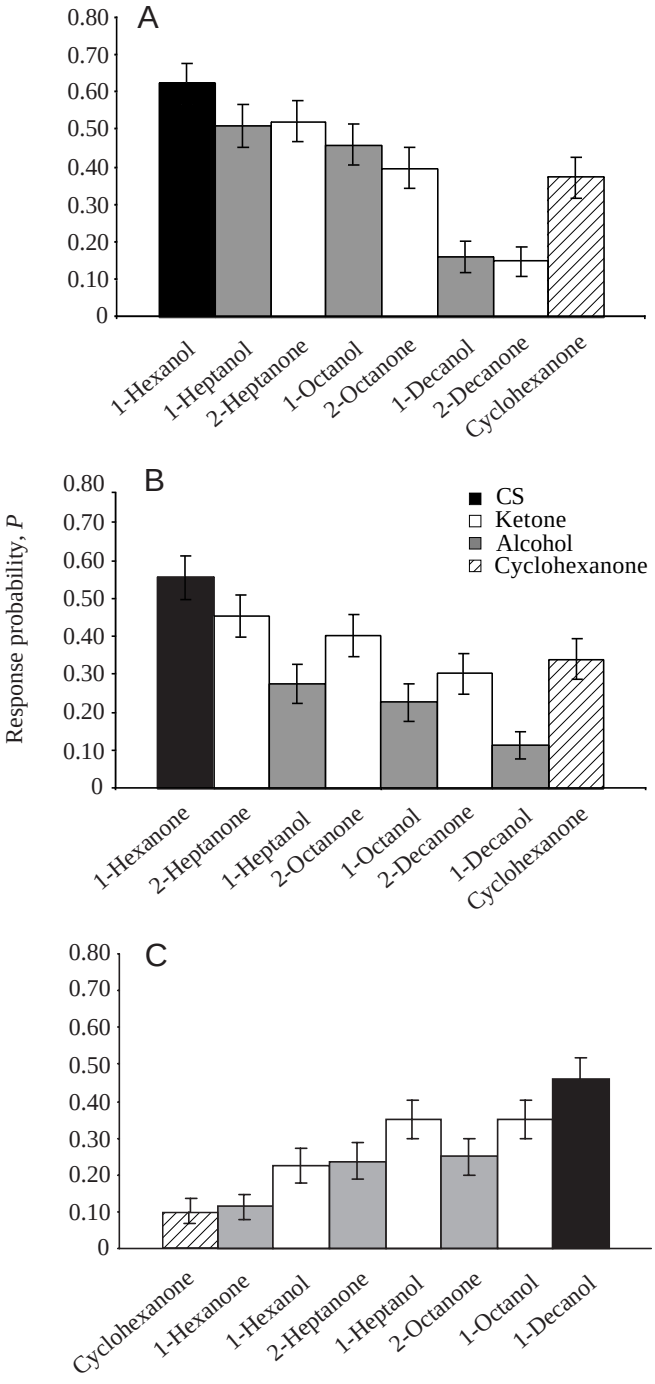

Fig. 2. Response probabilities *P* for the 2 h post-test of generalized response to the conditioning odour (black, CS) and to test odours, color-coded by functional group; gray, alcohols; white, ketones; striped, cyclohexanone. Moths were conditioned to either 1-hexanol (A; *N*=80), 2-hexanone (B; *N*=80) or 1-decanol (*C*; *N*=80).

Table 2. *Results of GLM and regression analyses for experiment 1*

	Variable	d.f., d.d.f.	Type I ss	r^2	*F* value	*P*
A	Model, error	3, 635	15.88, 137.91	0.10	24.41	0.0001*
	Chain length	1	11.84	0.08	54.60	0.0001*
	Chain shape	1	4.01	0.02	18.53	0.0001*
	Functional group	1	0.97	0	0.11	0.7381
	FG×CL interaction	1	0.02	0	0.14	0.7110
	Regression: response=1.11+(–0.09)*CL					
B	Model, error	3, 635	10.44, 130.44	0.07	16.76	0.0001*
	Chain length	1	4.74	0.03	22.85	0.0001*
	Chain shape	1	1.96	0.01	17.99	0.0022*
	Functional group	1	3.73	0.03	9.44	0.0001*
	FG×CL interaction	1	0.01	0	0.04	0.8489
	Regression: response=0.72+(–0.04)*CL+(–0.17)*FG					
C	Model, error	3, 475	6.09, 93.08	0.06	10.38	0.0001*
	Chain length	1	4.45	0.05	22.78	0.0001*
	Chain shape	1	0.32	0	1.62	0.2039
	Functional group	1	1.32	0.01	6.76	0.0096*
	FG×CL interaction	1	0.03	0	0.14	0.7072
	Regression: response=–0.03+(0.05)*CL+(–0.12)*FG					

The conditioning odour was (A) 1-hexanol (B) 2-hexanone and (C) 1-decanone.

*P<0.01.

In all models, chain length (CL) codes for the number of carbon units on each molecule, ranging from 6 to 10.

Chain shape (CS) was treated as a categorical variable and refers to whether the carbon side chain was straight or cyclic.

Functional group (FG) was treated as a continuous variable, coded as 0 for odours within the same functional group as the conditioning odour and 1 if not.

A,B,C refer to Fig. 2A,B,C, respectively.

We also conditioned the moths to 1-decanol (Fig. 2C) in order to assess the likelihood that odour volatility affected the probability of response. The mean responsiveness in the test phase was lower for both the conditioning and test odours in this group relative to the earlier two groups. Given that all three groups produced highly similar acquisition curves in the conditioning phase (see Fig. 1), this indicates that exposure to 1-decanol resulted in less retention of the conditioned response. However, the magnitude of the slope for the chain length variable for these three groups was roughly the same as before *(*–0.09, –0.04 and +0.05 for groups 1, 2 and 3, respectively). Note in Table 2C that the parameter estimate for chain length is positive because generalization tests were arranged in order of decreasing chain length, whereas in groups 1 and 2 the tests were in the direction of increasing chain length. The absolute value of the slope for the chain length variable in the 1-decanol group (0.05) falls between the absolute value of the slopes for chain length in the other two groups. This is particularly important as it indicates that odour volatility cannot account for the change in response probability that occurs with increasing differences in the carbon chain length between the conditioning odour and the test odours.

There was also a significant functional group effect when moths were conditioned to 1-decanol (Table 2C), whereas in group 1, when moths were conditioned to 1-hexanol, this effect was not significant. In the 1-decanol group, switching from an alcohol to a ketone resulted in a 12% decrease in the probability of a generalized response. However, after accounting for variance attributable to chain length and functional group, chain shape was not significant and this may be attributable to a floor effect for the behavioural response. The mean initial response probabilities for the conditioning odours in Fig. 1 range between 0.12 and 0.08 and the mean response probability for cyclohexanone in Fig. 2C was 0.10, which suggests that the response probability was at or near the baseline response probability for cyclohexanone. Our previous work with cyclohexanone further supports this notion (Daly and Smith, 2000). Finally, in all three groups we failed to find a significant interaction between carbon chain length and functional group (Table 2), which indicates that these variables function independently of one another.

Differential conditioning produces shifts in the generalization gradient

In the second series of experiments moths were differentially reinforced to two odours that varied in degree of relatedness. The first two groups received differential conditioning to two

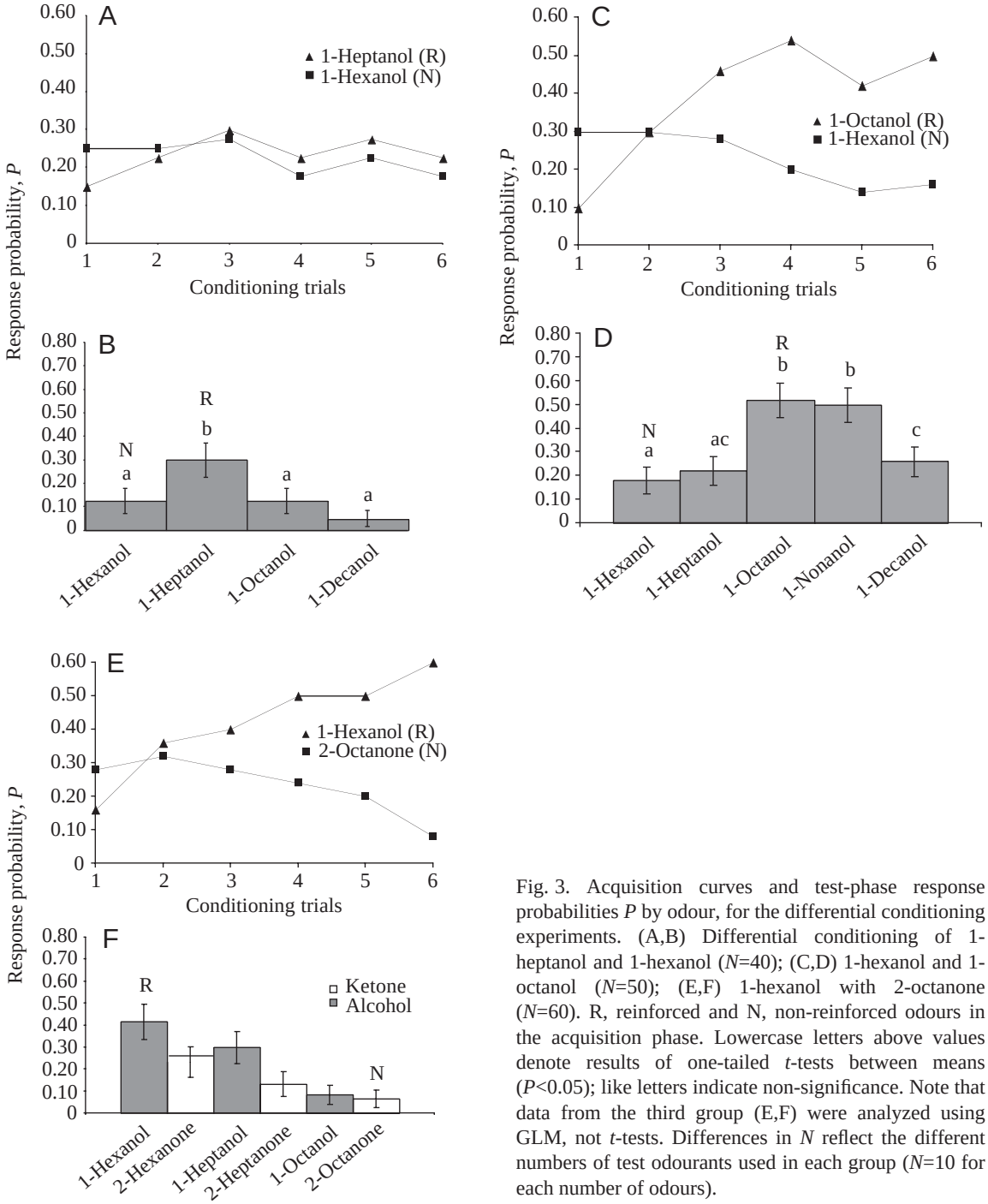

Fig. 3. Acquisition curves and test-phase response probabilities *P* by odour, for the differential conditioning experiments. (A,B) Differential conditioning of 1-heptanol and 1-hexanol (*N*=40); (C,D) 1-hexanol and 1-octanol (*N*=50); (E,F) 1-hexanol with 2-octanone (*N*=60). R, reinforced and N, non-reinforced odours in the acquisition phase. Lowercase letters above values denote results of one-tailed *t*-tests between means ($P<0.05$); like letters indicate non-significance. Note that data from the third group (E,F) were analyzed using GLM, not *t*-tests. Differences in *N* reflect the different numbers of test odourants used in each group (*N*=10 for each number of odours).

alcohols that differed in carbon chain length, whereas in the third group the two odours varied in carbon chain length and functional group. Moths were then tested with an array of odours to determine how differential conditioning affected the generalization gradient. Fig. 3A shows the mean response probabilities during the conditioning phase for 1-heptanol (R) and 1-hexanol (N). Note that there is very little divergence in mean response probability between the reinforced and non-reinforced odours over conditioning trials, indicating that moths had difficulty in discriminating between these two odours. There was a relatively low mean response probability to the conditioning odour ($P=0.30$) and other odours in the test phase (Fig. 3B). One-tailed *t*-test comparison of means indicates that there was a significant decrease in response probability from the conditioning odour to all of the test odours. But the lack of a significant difference between 1-hexanol and 1-octanol indicates that the generalization gradient drops off symmetrically on either side of the forward paired odour (1-hexanol).

In contrast, when moths were differentially conditioned to

Table 3. *Results of GLM and regression analyses for experiment 2*

Variable	d.f., d.d.f.	Type I ss	r^2	*F* value	*P*
Model, error	2, 357	5.09, 53.69	0.09	16.94	0.0001*
Chain length	1	3.75	0.07	24.93	0.0001*
Functional group	1	1.34	0.01	8.94	0.0030*
FG×CL interaction	1	0.42	0.01	2.78	0.0961

Regression: Response=1.14+(-0.13)*CL+(-0.12)*FG

The conditioning odours in this case were 1-hexanol (reinforced) and 2-octanone (not reinforced).

*$P<0.01$.

Chain length (CL) codes for the number of carbon units on each molecule, ranging from 6 to 10.

Chain shape was treated as a categorical variable and refers to whether the carbon side chain was straight or cyclic.

Functional group (FG) was treated as a continuous variable, coded as 0 for odours within the same functional group as the reinforced odour and 1 if not.

1-octanol (R) and 1-hexanol (N), there was a rapid divergence in response probabilities over conditioning trials (Fig. 3C). There was also a negative skew in the generalization gradient in the test phase (Fig. 3D), as indicated by the significant difference between 1-heptanol and 1-octanol and the lack of a significant difference between 1-octanol and 1-nonanol.

Moths differentially conditioned to 1-hexanol (R) and 2-octanone (N) also displayed a rapid divergence in response probability over conditioning trials (Fig. 3E). Fig. 3F displays the mean response probabilities for this group during generalization testing. Statistical results (Table 3) indicate a significantly negative effect of chain length. Slope data suggest that response probability dropped off by 13 % in this group, a sharper drop than that observed in experiment 1. This could be attributable to the influence of the non-reinforcement of odour N. The interaction between chain length and functional group was again not significant (Table 3), suggesting that these dimensions are independent. Finally, there was a functional group effect, which produced an additional 12 % drop when switching from alcohols to ketones. This is in contrast to the result in experiment 1, when we forward-paired 1-hexanol alone (Fig. 2A). Thus this drop in response across functional group is probably attributable to the inhibitory generalization gradient produced by presenting, but not reinforcing, 2-octanone during conditioning.

Discussion

The goal of this study was to use a behavioral assay to investigate how odours are coded in a model olfactory system represented by the sphinx moth *M. sexta*. Using a behavioural assay to elucidate the degree to which moths can make distinctions between odours that vary according to one or more molecular dimensions, we are able to deduce how those odours are represented in the nervous system. We can now draw inferences about the dimensionality of odour space and tuning of the olfactory system. Based on this analysis we conclude that the moth olfactory system distinguishes chain length, shape and functional group for the odourants used in our study. Ultimately, this analysis has provided a number of hypotheses for physiological investigations of the neural representations of these same odourants.

We have investigated geometry-based dimensions specifically with regard to how they influence the generalization of a conditioned response. We have found that as the absolute value of the difference in the carbon chain length between the conditioning and test odours increases there is a corresponding decrease in the probability that the test odour will elicit a response. This pattern was evident for all three conditioned odourants. It did not matter whether we conditioned to short chain molecules and tested generalization to longer chain lengths or *vice versa*. Statistical analyses revealed that addition or deletion of one carbon (methylene) unit produced a 4–9 % decrease in response probability. This rate of decrease was consistent at least across several carbon units away from the conditioned odourant. Thus we can conclude that, all other aspects of the molecule being equal, the length of the carbon chain is a predictable odour dimension that is represented by the moth olfactory system.

In contrast, generalization of a conditioned response across molecules that contain different functional groups appears to be asymmetric. That is, we observed a significant functional group effect in two of three cases. When conditioned to 2-hexanone or to 1-decanol, moths generalized more to molecules from the same functional group. In these two cases, a change in functional group produced a 12–17 % decrease in the response for any given carbon chain length. Furthermore, the effect of chain length was not influenced by a change in functional group; that is, these two dimensions do not interact. Once the effect of functional group is taken into account, the decrease across chain length within the different functional groups was the same. This lack of interaction, at least for these conditioning odourants, suggests that chain length and functional group are coded independently by the moth olfactory system.

In the remaining case, and in contrast to the results for 1-decanol, when moths were conditioned to 1-hexanol they generalized equally to alcohols and ketones of equivalent carbon chain length. This lack of differential generalization across functional group probably indicates that alcohols and ketones are perceptually similar, but not necessarily identical, given conditioning to 1-hexanol. Moths can still discriminate alcohols from ketones, as revealed by our discrimination conditioning experiment in which 1-hexanol and 2-octanone were differentially reinforced. However, in this second case, we must view the significant functional group effect in light of the moth's experience with 2-octanone, not 1-hexanol. In spite of this asymmetry in generalization from alcohols to ketones, however, these results imply that there is a functional group dimension to odour coding that is independent of carbon chain length.

We also used discrimination conditioning to further investigate the chain length and functional group dimensions. Under this condition, as in the purely excitatory conditioning in the first experiments, the reinforced odour (R) would be expected to generate a locus of excitation that would overlap with neural representations of similar odours in a graded fashion to produce the generalization gradients described for chain length, shape and functional group. Likewise, the non-reinforced odour (N) would be expected to produce a locus of inhibition that would overlap with neural representations of similar odours in a graded manner, thus producing and inhibitory generalization gradient. Theory predicts that if these two gradients overlap on a single dimension they will summate with one another, producing the peak shift effect (Spence, 1937). Peak shift experiments similar to those described here have been used to assess stimulus dimensionality of the visual system of vertebrates (Hanson, 1959; Mood et al., 1991) and, more recently, to understand processing in the auditory cortex (Weinberger, 1998).

Our results indicate that these two gradients may indeed summate to produce shifts in the generalization gradient when the peaks of the excitatory and inhibitory gradients were far enough apart to give a perceivable difference between the two stimuli. In that case the summed excitatory gradient should shift away from the locus for the positive peak and in the direction opposite to that of the negative peak. We observed a peak shift in our data when we conditioned subjects to discriminate between two alcohols that differed by 2 carbon units, and again when we conditioned subjects to discriminate an alcohol from a ketone. In the former case, the generalization gradient still revealed a strong response to 1-octanol, which was the reinforced conditioned stimulus. But there was an equally strong response to 1-nonanol, which has a chain length that is longer than 1-octanol by one carbon unit. Given the results of experiment 1, we would have expected, based on the null hypothesis, a 9 % decrease in response from 1-octanol to 1-nonanol. Both odours, however, produced roughly the same response. Furthermore, we would have expected only a 9 % drop from 1-octanol to 1-heptanol, but we observed a 30 % decrease. This shift in symmetry of the generalization gradient implies that inhibition had accrued to the non-reinforced odourant, 1-hexanol, and that this inhibition then generalized along the chain length dimension to interact with the excitatory gradient around 1-octanol.

A peak shift was also observed when we conditioned subjects to discriminate 1-hexanol, which was reinforced, from 2-octanone, which was not reinforced. In this case the shift was indicated by the significant functional group effect, which was not observed when 1-hexanol was conditioned alone. As before, if inhibition had accrued to the ketone it would have resulted in a lowered response to the similar ketones as well as to alcohols in general, but the effect would have been greater with ketones because, as we have shown, conditioning to ketones produces a functional group effect. That there is still a chain length effect evident among the tested ketones reveals an overlap with the excitatory gradients produced by the alcohols. Furthermore, after accounting for the functional group effect imposed by the inhibitory conditioning of 2-octanone, the slope for chain length was steeper here than in experiment 1 where 1-hexanol was conditioned alone. This too suggests that the inhibitory gradient along the chain length dimension generalized from 2-octanone to both alcohols and ketones, as expected, and summated with the chain length gradient produced by 1-hexanol.

Following this logic (Spence, 1937), in cases where the two test stimuli are too similar, the inhibitory and excitatory gradients should almost completely overlap. The result should be a generalization gradient that is symmetrical, but the peak response should be reduced in magnitude to reveal a platy-kurtic or flat gradient. The results of differentially conditioning to 1-hexanol and 1-heptanol are characteristic of this prediction. Given the weak acquisition slope and the symmetrical but flattened generalization gradient, it would be reasonable to conclude that the two odours are not sufficiently distinct but the two gradients are still interacting along a single chain length dimension.

Extending the logic of Spence (Spence, 1937), if inhibitory and excitatory gradients are purely additive and linear, as indicated by our data, then the net slope for chain length should be predictable in experiment 2, where 1-hexanol and 2-octanone were differentially conditioned, by summing the absolute values of slopes for the chain length variables from the ketone and alcohol conditioning groups in experiment 1. The slopes for 1-hexanol and 2-hexanone, which are the most closely matched odours from experiment 1, equal 0.09 and 0.04 respectively (see Table 2A,B). In this case we only assume that the magnitude of the excitatory and inhibitory gradients would be the same for an odour, given the same number of conditioning trials. Because the gradient is linear we can estimate that the slope for 2-hexanone would approximate the slope for 2-octanone. What is surprising is that summation precisely predicts the net slope for chain length produced by differential conditioning of 1-hexanol and 2-octanone ($|\text{slope}|=0.13$; Table 3). While this is still speculative and requires a more direct empirical analysis, the finding supports the idea that the inhibitory and excitatory gradients are of the same general magnitude and that they summate in a purely additive fashion.

These data permit hypotheses that warrant further behavioural and physiological investigation into odour coding in the moth. Specifically, the neural representations of these alcohols and ketones should overlap in a manner that is consistent with our generalization data. This overlap could involve features of both spatial and temporal coding. Considerable evidence has accumulated to reveal that spatial codes are an important feature of both the vertebrate and invertebrate olfactory systems. In the antennal lobe of the honeybee, for example, spatially overlapping patterns of glomerular activation are correlated to stimulation with different odourants (Galizia et al., 1999). Physiological data from the rabbit olfactory bulb (Mori et al., 1992; Katoh et al.,

1993) also reveal spatial patterns of mitral cell activation that are distinct but overlap for different but related odourants. Changes in carbon chain length and function group, as we have done here, correlate to differences in the pattern of mitral cell activity, in that the overlap of activity patterns correlates to difference between molecules that are compared.

Studies of the locust (Laurent and Davidowitz, 1994; Laurent and Naraghi, 1994; Laurent, 1996; Wehr and Laurent, 1996) and honeybee (Stopfer et al., 1997) antennal lobes have also revealed a temporal component to odour coding. Stimulation with odour gives rise to regular low frequency oscillations in the local field potential measured in the projection fields of the antennal lobe projection neurons. Each cycle in the LFP oscillation is caused by a spatial array of activated projection neurons that show phase-locked oscillations in their membrane potentials and synchronization of action potentials. Each succeeding cycle is caused by a partially or completely different synchronized spatial array. Desynchronization of neurons by pharmacological treatment (Macleod and Laurent, 1996) impairs the ability of honeybees to make fine discriminations, for example, between 1-hexanol and 1-octanol, but it leaves intact the ability to discriminate odourants that are more distinct (Stopfer et al., 1997).

Therefore, both spatial and temporal coding features appear to be important for accurate neural representations of odourants. These codes must in some way represent information about the molecular features that we have determined to be important for odourants used in our study. Odourants with more similar chain length should show greater overlap in the spatial and/or temporal codes for those odourants in the antennal lobe. For a given chain length, ketones and alcohols should show some overlap because of their common chain length. It should also be noted that this overlap could arise in excitatory and/or inhibitory pathways, as indicated by our peak shift analyses. Using recently developed techniques for multi-site recording in the moth antennal lobe (Christensen et al., 2000) that can be coupled with olfactory conditioning (K. C. Daly, T. A. Christensen, V. M. Pawlowski, H. Lei, B. H. Smith and J. G. Hildebrand, manuscript submitted for publication) it should now be possible to evaluate these hypotheses and investigate how plasticity influences the neural representation for odourants in moths.

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