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Olfactory coding in *Drosophila* larvae investigated by cross-adaptation

Jennefer Boyle and Matthew Cobb*
Faculty of Life Sciences, The University of Manchester, Oxford Road, Manchester M13 9PT, UK
*Author for correspondence (e-mail: cobb@manchester.ac.uk)

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Summary

In order to reveal aspects of olfactory coding, the effects of sensory adaptation on the olfactory responses of first-instar *Drosophila melanogaster* larvae were tested. Larvae were pre-stimulated with a homologous series of acetic esters (C3–C9), and their responses to each of these odours were then measured. The overall patterns suggested that methyl acetate has no specific pathway but was detected by all the sensory pathways studied here, that butyl and pentyl acetate tended to have similar effects to each other and that hexyl acetate was processed separately from the other odours. In a number of cases, cross-adaptation transformed a control attractive response into a repulsive response; in no case was an increase in attractiveness observed. This was investigated by studying changes in dose–response curves following pre-stimulation. These findings are discussed in light of the possible intra- and intercellular mechanisms of adaptation and the advantage of altered sensitivity for the larva.

Key words: olfaction, *Drosophila melanogaster*, adaptation, maggot.

Introduction

Adaptation occurs when a continuous presentation of a sensory stimulus leads to a temporary decline in the organism's response to that stimulus. Adaptation can be considered as a form of neuronal plasticity (Colbert and Bargmann, 1995) and can be mediated by peripheral or central events. The molecular bases of peripheral olfactory adaptation in multicellular organisms appear to involve modulation of Ca^{2+} trafficking: short-term olfactory adaptation (measured in seconds) changes Ca^{2+} levels in the receptor cell, while longer-term stimulation (over minutes or hours) also induces changes in intracellular cGMP (Zufall and Leinders-Zufall, 2000). Mutant studies of long-term olfactory adaptation in *Drosophila melanogaster* and *Caenorhabditis elegans* have implicated inositol (1,4,5)-trisphosphate (Deshpande et al., 2000) and cGMP (L'Etoile et al., 2002), respectively. Although all studies of the molecular bases of adaptation in receptor neurons revolve around the role of calcium, its effect is complex ('two-faced'; Matthews and Reisert, 2003), showing both excitatory and inhibitory actions, affecting Cl^-, Na^+ and K^+ channels, and interacting with Calcium/calmodulin-dependent protein kinase (CaMK), cAMP and cGMP.

Intercellular processes such as lateral inhibition may also be involved in mediating long-term adaptation (Urban, 2002). This process might be relatively complex, involving positive and negative signals and interactions at various levels. A given olfactory receptor neuron can be both inhibited and stimulated, depending on the stimulus applied to it (Hallem et al., 2004; Oka et al., 2004), while higher processing units such as glomeruli can show both lateral excitation and lateral inhibition, producing a sharpening of the olfactory code (Schoppa and Urban, 2003).

Paradoxically, one of the effects of olfactory adaptation to a given odour can be to enable the organism to detect small changes in the levels of other substances (Kelling et al., 2002; Fain, 2003). This effect, coupled with the fact that in the natural world animals are often in the presence of continuous background odours (e.g. from food sources, nests or conspecifics), suggests that adaptation may play an important role in shaping and tuning the olfactory response. The precise processes that lead to such increased sensitivity through adaptation are not known but can be presumed to be consequences of some or all of the mechanisms outlined above.

Adaptation can be used as a tool to reveal the organisation of the organism's sensory response: if adaptation following stimulation with stimulus A leads to a decline in the response to both stimulus A and stimulus B, it can be concluded that some or all aspects of the sensory processing of the two stimuli are shared. This approach, known as cross-adaptation, has been employed in studies of chemosensation in a range of organisms, including bacteria (Gestwicki and Kiessling, 2002), lobsters (Daniel et al., 1994), mice (Kelliher et al., 2003), frogs (Takeuchi et al., 2003), houseflies (Kelling et al., 2002), *C. elegans* (Colbert and Bargmann, 1995), *Manduca sexta* (Dolzer et al., 2003) and *Drosophila* adults (de Bruyne et al., 1999) and larvae (Cobb and Domain, 2000).

A simple model of olfactory processing would predict that cross-adaptation should be reciprocal (i.e. pre-stimulation with

odour A affects odour B and *vice versa*); more complex models might predict an element of asymmetry – for example, a short-chain molecule could lead to adaptation in the processing pathways responsible for the response to a longer-chain molecule, but the reciprocal effect would not occur because the larger molecule would have low or no affinity with the receptors associated with the response to the smaller molecule. As these hypothetical examples imply, this simple technique has the added advantage of generating hypotheses about coding that can eventually be tested by more complex approaches, such as electrophysiology.

Fruit fly maggots combine the genetic manipulability of adult *D. melanogaster* with a substantially lower level of complexity. For example, the peripheral olfactory system of the larva consists of 21 receptor neurons, as against 1300 in the adult (Cobb, 1999). The larval olfactory receptor neurons project into the antennal lobe of the larval brain, where they each project to a single glomerulus in the antennal lobe, as in the adult brain and in vertebrates (Python and Stocker, 2002; Ramaekers et al., 2005). This combination of reduced receptor complexity and fundamental homology with more complex organisms, combined with a very limited behavioural repertoire and the ability to detect over 60 odours (Cobb, 1999), makes the larva a useful preparation for studying basic processes in olfactory coding.

The *Drosophila* genome is thought to contain around 60 olfactory receptor genes (Clyne et al., 1999; Vosshall et al., 1999), of which 13 are apparently not expressed in the adult (Robertson et al., 2003). It has long been argued that, in most organisms except *C. elegans*, only one type of receptor gene is expressed in each olfactory receptor neuron. However, the evidence for this 'rule' is less solid than initially appeared (Mombaerts, 2004) and it has recently been shown in *Drosophila* adults that one class of olfactory receptor neurons expresses more than one type of olfactory receptor (Goldman et al., 2005). This also appears to be the case in some *Drosophila* larval receptor neurons – a total of 23 *Or* genes have recently been reported to be expressed in the larva, for 21 olfactory sensory neurons (Kreher et al., 2005).

In a previous study, we used cross-adaptation to investigate olfactory coding of alcohols in *Drosophila* larvae (Cobb and Domain, 2000). That study suggested that a form of lateral inhibition – occurring either peripherally or centrally – underlay the ability of the maggot olfactory system to produce a variety of attractive and repulsive responses to different alcohols. Here, we use adaptation to study the olfactory responses of *Drosophila* larvae to another homologous series of ecologically meaningful odours – short-chain acetic esters or aliphatic acetates. The genetic bases of larval responses to these odours have been studied and have been shown to include factors on all major chromosomes, with specific and separate anosmias to pentyl acetate and hexyl acetate, indicating that these odours can be distinguished by larvae (Cobb and Dannet, 1994). The present study not only provides information about the organisation of the olfactory response to these important odours but it also enables us to test the generality of the lateral inhibition model we developed for the coding of alcohols.

Materials and methods

Preparation of larvae

First instar *Drosophila melanogaster* Canton–S larvae were reared for 20–24 h at 25°C on a yeast paste prior to testing. Before testing, larvae were washed from the yeast paste using distilled water, transferred to a clean Petri dish (2.5% agar) and starved for 1 h; immediately before testing they were washed from the dish and dried. In tests involving adaptation (see below), maggots were cleaned and starved for the appropriate amount of time prior to pre-stimulation, such that the total amount of time between removal from the yeast paste and testing was 60 min.

Olfactory tests

Olfactory responses were measured following Cobb and Domain (2000). Briefly, approximately 50 larvae were placed in the centre of a Petri dish filled with 2.5% agar. Two 12.7 mm-diameter filter papers (one for the test odour, one as a control) were positioned on opposite sides of the dish, on the lid of a small micro-centrifuge tube, which prevented larvae from coming into contact with the test odour, thereby excluding gustatory effects. Standard test odour volumes (1 μl for butyl…heptyl acetate; 2.5 μl for all other odours) were applied to the filter paper using a micro-pipette or a micro-syringe. For dose–response curves, test volumes were varied as described in the text. All chemicals were used undiluted and were Merck analysis grade. After 5 min, the number of larvae on the control and odour sides, and the number of 'non-choosers' in a 5 mm-wide central strip, were recorded. A response index (RI) was calculated: $RI=[(n_{odour}-n_{control})/n_{total}]\times 100$, which varies between –100 (total repulsion) and +100 (complete attraction). During testing, maggots will initially disperse at random before meeting the diffusing odour and moving towards it or away from it, depending on whether they are attracted or repulsed (Cobb, 1999). Some maggots fail to 'choose' by either remaining in the start circle or finding themselves in the central strip at the time the number of maggots is counted. The 'no-choice' zones make up ~11% of the total surface area of the Petri dish. Tests where >30% of maggots failed to choose were discarded; these very rare tests always involved situations in which a relatively high proportion of maggots failed to leave the start circle, normally because they had been damaged while being collected from the agar plate. In control responses to the seven aliphatic acetates tested here, the mean percentage of maggots failing to choose was 17.45±1.14%. In the case of tests following auto-adaptation, the figure was slightly lower (14.92±0.87%), indicating that adaptation did not in any way reduce the mobility of the larvae. In both cases, this figure is higher than the surface area of the no-choice zone (11%); this is due to the fact that in virtually all tests some larvae did not leave the start circle. 6–22 replicates were performed for each

test; data were either pooled to form a single overall index that could be tested using a contingency test (for defining the experimental conditions for adaptation – see below) or mean response indices and standard errors were calculated to provide a measure of the variability between dishes and allow for testing by analysis of variance (ANOVA) or *t*-test. This test does not involve any 'stampede effect'; the response of each individual larva is independent from the behaviour of those around it (M. Kaiser and M. Cobb, manuscript in preparation). The response index is a sensitive and robust phenotype that has enabled genetic factors controlling quantitative variation in the olfactory response to be localised (Cobb and Dannet, 1994).

Adaptation

After having been washed from the yeast, larvae were placed in a clean agar-covered Petri dish and pre-stimulated with one of the acetic esters: the odour was loaded onto a filter disc placed on the lid of a micro-centrifuge tube. Pre-stimulation volume/duration combinations for each odour that produced auto-adaptation were determined in preliminary experiments. Auto-adaptation was defined as the total distribution of larvae that did not differ significantly from the distribution observed in control tests without an odour stimulation, when compared using χ^2. Pre-stimulation combinations were: methyl acetate, 40 μl, 20 min; ethyl acetate, 40 μl, 25 min; propyl acetate, 35 μl, 25 min; butyl acetate, 25 μl, 15 min; pentyl acetate, 10 μl, 10 min; hexyl acetate, 10 μl, 15 min; heptyl acetate, 10 μl, 15 min. In the case of hexyl acetate, control responses were not significantly different from 0, so it was impossible to detect auto-adaptation. For this odour, the same volume/time combination as heptyl acetate was chosen. Pre-stimulation began at an appropriate point such that the total time between being washed from the yeast and being tested was 60 min.

Results

As expected (Cobb and Dannet, 1994), unadapted larvae were strongly attracted to ethyl and pentyl acetate and repulsed by heptyl acetate (Fig. 1). Following pre-stimulation, larvae showed auto-adaptation to each odour, as shown by non-significant χ^2 tests comparing the observed distribution of larvae with those observed following control tests with no odour (data not shown). Adaptation is a temporary effect: after 60 min recovery in clean air following pre-stimulation, the RI of larvae had returned to approximately normal for all seven odours (Fig. 1). An ANOVA comparing the olfactory responses of control and recovered larvae showed no treatment effect if ethyl acetate was excluded from the data ($F_{1,99}$=2.03, P=n.s.) and no significant treatment × odour interaction ($F_{5,99}$=0.91, P=n.s.). Ethyl acetate, which was highly attractive following recovery (RI=46.2±4.33), induced significantly weaker attractive responses compared with control levels (t_{19}=5.87, P=0.0001).

Table 1 shows the mean olfactory response of larvae tested with C2–C8 acetic esters following adaptation with each of these odours. The patterns of responses of each odour to pre-

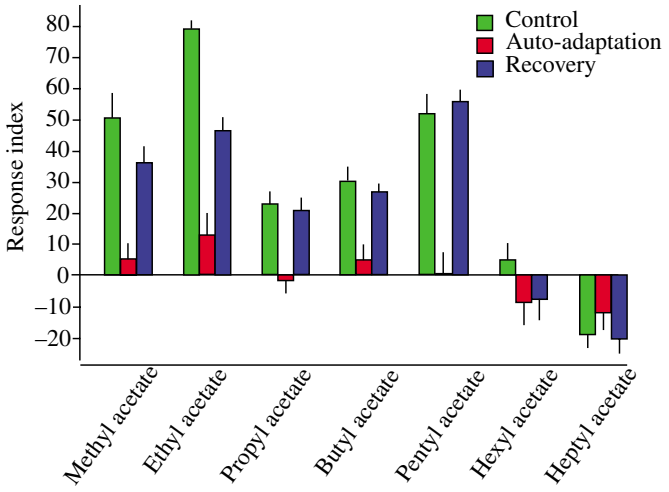

Fig. 1. Mean olfactory responses to seven homologous aliphatic acetates (methyl…heptyl acetate) under control conditions (green), following auto-adaptation (red) and after 60 min recovery from auto-adaptation (blue). Error bars show S.E.M.

stimulation with each of the seven aliphatic acetates are unique: this suggests that larvae not only detect but also discriminate these odours. Secondly, the patterns of adaptation and non-adaptation are not symmetrical, nor do they show a clear change with carbon number. This indicates that the sensory processing of these relatively simple homologous acetic esters does not function according to simple linear or arithmetical rules.

To test for significant effects in Table 1, we used *t*-tests to compare the responses to each test odour following pre-stimulation with the control responses to that odour. In order to take account of the multiple comparisons of each test odour, Bonferroni's correction was applied (a significance threshold of P=0.007). Fig. 2 shows a graphical representation of the results of this analysis of Table 1: coloured blocks represent significant changes in the response to a test odour (in all cases, the change was a significant reduction in attractiveness); a blank block indicates no significant change compared with control levels. To measure the nature of the reduction in attractiveness, multiple one-sample *t*-tests were carried out on the effects of pre-stimulation for each test odour, comparing the observed results with a theoretical score of 0 (indifference, i.e. no mean attraction or repulsion). Again, Bonferroni's correction was applied. Purple blocks indicate that the behavioural response was indifferent (no significant difference from zero as measured by a one-sample *t*-test). Red blocks indicate that the test odour was still significantly attractive; blue blocks indicate that the test odour was significantly repulsive.

The two odours at either end of the molecular range studied here – methyl and heptyl acetate – differ from all the others. Test responses to methyl acetate were affected only by pre-stimulation with methyl acetate. Test responses to heptyl acetate were not significantly affected by pre-stimulation with

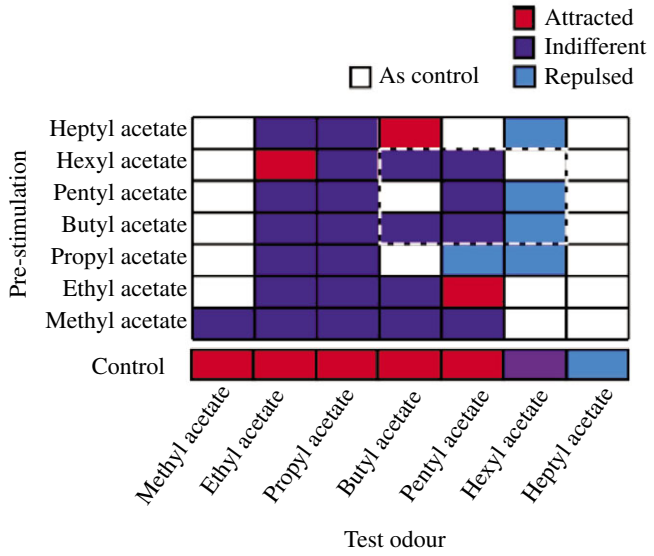

Fig. 2. Graphical representation of analysis of data in Table 1. The response to each test odour following pre-stimulation was compared with the control level using *t*-tests and Bonferroni's correction for multiple comparisons (see text for details). Responses that were significantly different from control levels are indicated in colour. Red indicates that the response was significantly weaker than control levels of attraction but was still attractive. Purple indicates that the mean response was not significantly different from zero, as tested by a one-sample *t*-test, and is therefore described as 'indifferent'. Blue indicates that the repulsive response was significantly different from zero. A blank rectangle indicates that the response was not significantly different from the control levels. Control responses were all tested using one-sample *t*-tests and are indicated as being significantly attractive (red), not significantly different from zero (i.e. indifferent – purple) or significantly repulsive (blue). The reciprocal crosses between butyl, pentyl and hexyl acetate, discussed in detail in the text, are highlighted with a broken box.

any odour, suggesting that this odour is processed separately from the other acetic esters studied here.

Test responses to ethyl acetate were completely abolished by pre-stimulation with all odours except hexyl acetate, which still significantly reduced the attractiveness of this test odour. Conversely, pre-stimulation with ethyl acetate led to full cross-adaptation only in the cases of propyl and butyl adaptation, with a significant reduction in the response to pentyl acetate. Test responses to propyl acetate were abolished by pre-stimulation with all seven aliphatic acetates. In the cases of pre-stimulation with hexyl and heptyl acetate, the *t*-test comparisons with control responses were not significant with

Table 1. *Effects of pre-stimulation with seven acetic esters (C3–C9) on olfactory responses to these odours*

	Test odour						
Pre-stimulation	Methyl acetate	Ethyl acetate	Propyl acetate	Butyl acetate	Pentyl acetate	Hexyl acetate	Heptyl acetate
Heptyl acetate	59.50 (4.04)	20.57 (7.02)	7.70 (5.06)	19.00 (5.42)	46.67 (5.58)	–23.47 (4.09)	–12.13 (4.59)
Hexyl acetate	36.70 (6.98)	32.10 (4.75)	5.27 (4.20)	5.57 (4.08)	9.23 (3.15)	–8.78 (7.11)	–20.57 (9.82)
Pentyl acetate	36.24 (5.98)	1.43 (3.15)	–5.06 (3.16)	31.89 (5.86)	0.28 (6.72)	–35.83 (2.49)	–20.92 (6.25)
Butyl acetate	29.60 (6.56)	3.07 (5.12)	–3.84 (8.66)	4.67 (5.16)	–19.58 (6.10)	–32.56 (5.03)	–30.20 (4.96)
Propyl acetate	30.59 (5.86)	12.16 (6.38)	–1.84 (4.24)	34.77 (5.16)	–25.48 (5.02)	–22.09 (6.44)	–25.69 (8.45)
Ethyl acetate	41.64 (6.06)	12.61 (7.06)	–6.25 (3.83)	3.34 (3.45)	16.44 (2.49)	12.90 (13.45)	–24.48 (5.90)
Methyl acetate	5.11 (5.03)	6.43 (5.93)	1.33 (5.67)	12.41 (4.24)	8.18 (7.46)	–9.33 (5.45)	–15.64 (4.13)
Control	50.27 (8.04)	78.78 (2.73)	22.61 (2.71)	40.06 (4.36)	51.51 (6.18)	4.76 (5.38)	–20.51 (4.16)

Responses are given as mean response indices; standard errors are given in parentheses. Auto-adaptation data are shown boxed, for the sake of clarity. The broken box highlights the nine crosses between butyl…hexyl acetate, discussed in detail in the text.

Bonferroni's correction (t_{22}=2.74, P=0.011; t_{22}=2.25, P=0.035, respectively), but an inspection of Table 1 shows that the responses to propyl acetate following pre-stimulation are approaching zero. The existence of a significant reduction in the response to propyl acetate following pre-stimulation with hexyl and heptyl acetate was confirmed by comparison of the total frequencies of attracted, repulsed and non-choosing larvae in control and pre-adapted conditions (hexyl χ^2=15.61, d.f.=2, P=0.0004; heptyl χ^2=24.30, d.f.=2, P=0.0001). Pre-stimulation with propyl acetate had varying effects: it abolished responses to ethyl and propyl acetate, had no effect on butyl acetate and rendered pentyl acetate and hexyl acetate repulsive.

Butyl acetate and pentyl acetate induced similar effects: their control responses were not significantly different (t_{22}=1.52, P=n.s.) and, if the striking results of their reciprocal cross-adaptation were excluded (butyl–pentyl=–19.58; pentyl–butyl=31.89; see below), the two odours had identical effects when they were used as pre-stimulation. However, responses to these odours showed different effects following pre-stimulation with heptyl acetate (no effect on pentyl acetate, significantly reduced attraction for butyl acetate) and propyl acetate (no effect on butyl acetate, significant repulsion for pentyl acetate). The indifferent response to hexyl acetate under control conditions was transformed into a significantly repulsive response following pre-stimulation with propyl…pentyl acetate and heptyl acetate.

Some of the most intriguing results are to be found in the nine cells containing the test results for butyl, pentyl and hexyl acetate, following pre-stimulation with the same three acetates (broken boxes in Table 1 and Fig. 2). Each of the three pairs of cross-adaptation combinations (on either side of the leading diagonal) shows non-reciprocal cross-adaptation. Particularly striking are the negative responses induced by testing with pentyl and hexyl acetate (the control tests are strongly positive and indifferent, respectively) – similar results were found following pre-stimulation with propyl acetate.

Testing for changes in sensitivity

In many sensory modalities, stimuli that are attractive at low doses can become repulsive at a high dose (think of spilling a bottle of perfume): repulsive responses observed following adaptation may be due to changes in larval sensitivity to the test odour. Fig. 3 shows theoretical curves illustrating this hypothesis. Fig. 3A shows the effect of increased sensitivity in a system with a linear dose–response curve: low doses that induce no response in controls show a response after treatment. However, in a system in which high doses induce a negative response (Fig. 3B), it is possible that a dose that produces an attractive dose in control conditions will induce a repulsive response after treatment (arrow on Fig. 3B).

To test this hypothesis, we studied the responses of larvae to varying volumes of butyl acetate, pentyl acetate and hexyl acetate (see Materials and methods for details), following pre-stimulation with each of these three acetates. For the sake of clarity, the data for auto-adaptation (Fig. 4) and cross-adaptation (Fig. 5) are presented separately.

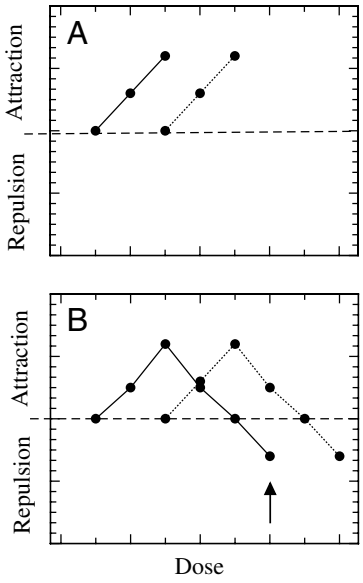

Fig. 3. Theoretical effect of an increase in sensitivity on dose–response curves. (A) A system showing a linear dose–response curve. (B) A system showing a negative response at higher doses. Dotted lines, control; solid lines, after treatment. The arrow indicates the dose at which an attractive dose becomes repulsive after treatment.

Larvae showed qualitatively different responses to auto-adaptation with each of the three odours tested: in the case of butyl acetate (Fig. 4A), the auto-adapted curve is significantly lower than the control responses ($F_{1,74}$=124.45, P<0.0001). Pentyl acetate (Fig. 4B) showed auto-adaptation at all doses tested ($F_{4,46}$=1.099, P=n.s.), while hexyl acetate (Fig. 4C) showed no change compared with control levels ($F_{1,94}$=0.051, P=n.s.). In no case did the curves change as predicted in Fig. 3.

Following cross-adaptation, larvae were consistently less attracted to the stimulus odours, and in many cases the response became repulsive, but, again, none of the curves resembled those predicted in Fig. 3. Pre-stimulation with both pentyl acetate and hexyl acetate produced a significant reduction in the response to butyl acetate with increasing test dose (Fig. 5A; $F_{4,39}$=29.202, $F_{4,42}$=7.044, respectively, P<0.001), but the effect of pentyl acetate was not significantly different from auto-adaptation ($F_{4,70}$=1.658, P=n.s.). With the exception of the final 10 μl test, the dose–response curve to pentyl acetate (Fig. 5B) showed a significant change with concentration following pre-stimulation with butyl acetate and hexyl acetate ($F_{3,58}$=4.333, P=0.008). The dose–response curves to hexyl acetate (Fig. 5C) showed a lower overall response after pre-stimulation with butyl acetate and pentyl acetate, in particular at 1 μl. The response was significantly lower after pre-stimulation with pentyl acetate compared with butyl acetate ($F_{1,48}$=11.32, P=0.0015), but cross-adaptation curves showed the same dose–response interaction ($F_{3,48}$=1.55, P=n.s.).

Cross-adaptation between functional groups

The effect of pre-stimulation with acetic esters on the

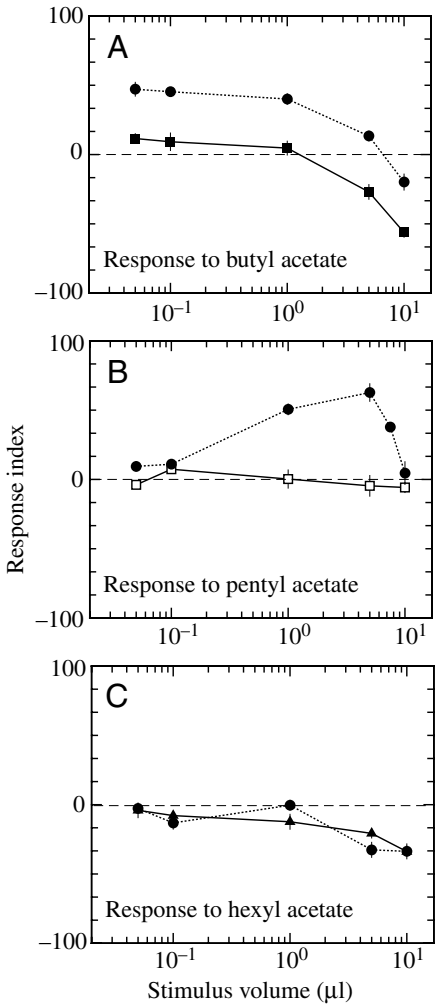

Fig. 4. Dose–response curves to test doses of three aliphatic acetates (A, butyl; B, pentyl; C, hexyl), showing mean response indices (N=8–16) and standard errors (error bars are sometimes smaller than the symbols used in the graph). Dotted line, control (no pre-stimulation). Solid line, following pre-stimulation with the same odour as the test odour. For full details, see text.

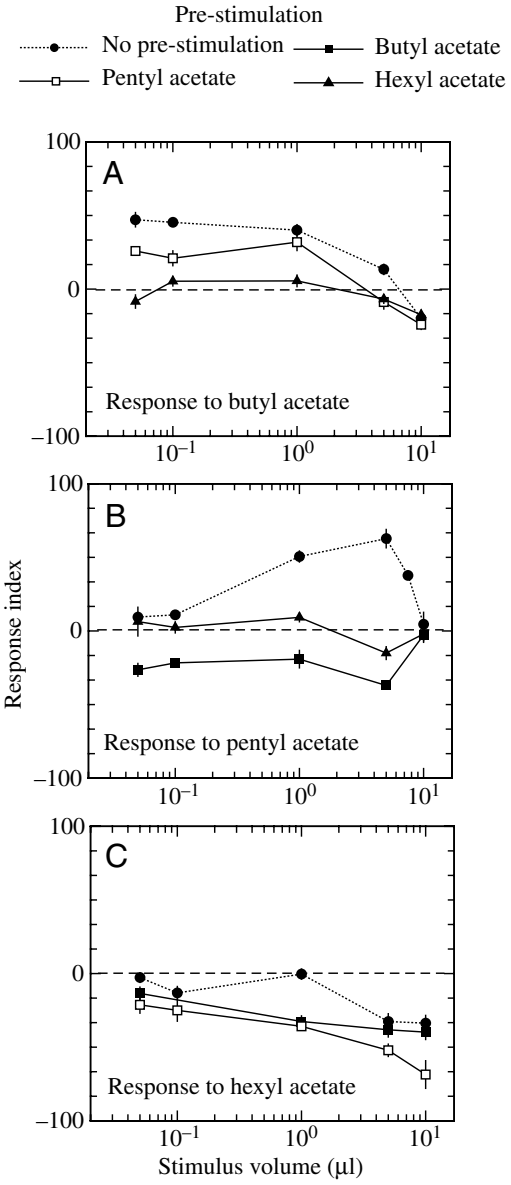

Fig. 5. Dose–response curves to test doses of three aliphatic acetates (A, butyl; B, pentyl; C, hexyl), showing mean response indices (N=8–16) and standard errors (error bars are sometimes smaller than the symbols used in the graph). Dotted line, control (no pre-stimulation); filled squares, following pre-stimulation with butyl acetate; open squares, following pre-stimulation with pentyl acetate; triangles, following pre-stimulation with hexyl acetate. For full details, see text.

olfactory response to odorants with other functional groups was tested with six alcohols (butanol…nonanol) and three acids (heptanoic, octanoic and nonanoic). Table 2 gives the mean response indices for these tests; as for Table 1, the data were compared using multiple t-tests for each test odour, using Bonferroni's correction (raising the significance level to P=0.017). Fig. 6 shows a graphical representation of the results of these tests. Full cross-adaptation was observed in the case of methyl acetate, which abolished the response to three of the alcohols tested here (butanol, pentanol and hexanol) and to heptanoic and octanoic acid. Pre-stimulation with ethyl acetate had no significant effects on the responses to any of the odours studied in this experiment. Strikingly, adaptation with propyl acetate induced a significant repulsive response to nonanoic acid, showing that larvae can detect this odour and will respond to it under certain circumstances.

Discussion

This study was carried out in order to provide some insight into the mechanisms of olfactory coding in *Drosophila* larvae, using adaptation as a behavioural probe into the function of a simple neural network. The most striking finding has been to alter our view of adaptation itself. Our data show that there are at least two ways that continual presentation of an olfactory stimulus can temporarily abolish the response to that odour: through adaptation (e.g. pentyl acetate auto-adaptation;

Table 2. *Mean olfactory responses to six alcohols (butanol…nonanol) and three acids (heptanoic, octanoic and nonanoic acid) following pre-stimulation with methyl, ethyl and propyl acetates*

	Test odour								
Prestimulation	Butanol	Pentanol	Hexanol	Heptanol	Octanol	Nonanol	Heptanoic acid	Octanoic acid	Nonanoic acid
Propyl acetate	27.06 (6.77)	27.51 (6.04)	32.31 (4.37)	24.36 (6.84)	–6.94 (6.09)	–39.60 (5.00)	33.77 (3.42)	31.84 (3.69)	–29.59 (2.81)
Ethyl acetate	33.48 (5.28)	35.88 (3.48)	39.52 (4.72)	26.70 (4.96)	–16.67 (2.57)	–43.94 (3.97)	27.42 (4.00)	25.66 (3.60)	4.44 (3.93)
Methyl acetate	0.11 (3.49)	10.98 (3.06)	11.04 (4.17)	45.56 (2.58)	–3.03 (3.05)	–42.18 (3.25)	–5.02 (6.07)	–8.89 (2.74)	5.48 (5.29)
Control	40.45 (6.20)	38.61 (10.40)	41.21 (2.12)	32.60 (5.33)	3.14 (5.18)	–48.51 (2.68)	36.58 (5.88)	32.61 (4.75)	12.01 (5.74)

Standard errors are given in parentheses. For full details of pre-stimulation procedures and test doses, see Materials and methods.

Fig. 4B) or by a change in sensitivity (e.g. butyl acetate auto-adaptation; Fig. 4A). Alterations in sensitivity appear to explain the extreme examples of non-reciprocal cross-adaptation observed here, where odours that induced attraction or indifference produced a repulsive response following cross-adaptation (Fig. 5B,C). It is striking that in none of the 76 pre-stimulation/test combinations studied here, nor in any of the 44 dose–response comparisons, did pre-stimulation lead to a significant *increase* in the attractiveness of the test odour. This fact is telling us something about the mechanisms involved in mediating the olfactory response after pre-stimulation.

Both this decline in attractiveness and the overall phenomenon of cross-adaptation could be an example of receptor cross-talk, where stimulation of one class of receptor leads to an alteration (generally a potentiation) of the response of another receptor class (Hill, 1998). Cross-talk often involves protein phosphorylation mediated by G-protein coupled receptor kinases within a given neuron (Vasquez-Prado et al., 2003), such as those implicated in olfactory adaptation in *C. elegans* (L'Etoile et al., 2002). This hypothesis raises three possibilities, which are not mutually exclusive:

(1) there may be more than one class of odour receptor on some or all larval receptor neurons responsible for detecting the odours studied here;

(2) intracellular interactions between receptor molecules from the same receptor class may lead to a change in the sensitivity to the ligand(s) for those receptors; this would explain the changes in response levels following auto-adaptation reported in Fig. 4A;

(3) finally, the effect may also be mediated by intercellular interactions, perhaps produced by a network similar to that proposed by Cobb and Domain (2000).

'Classic' receptor cross-talk would be expected to produce a clear leftward shift in dose–response curves, resulting in increased sensitivity (e.g. Fig. 3B), or an increase in the maximal response of the system (Selbie and Hill, 1998).

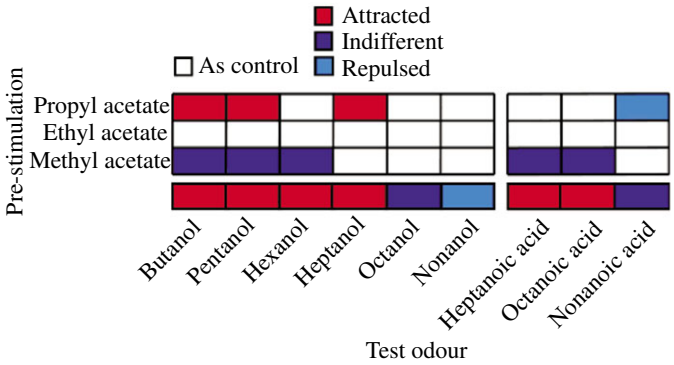

Fig. 6. Graphical representation of statistical analysis of the data in Table 2. The response to each test odour following pre-stimulation was compared with the control level using *t*-tests and Bonferroni's correction for multiple comparisons (see text for details). Responses that were significantly different from control levels are indicated in colour. Red indicates that the response was significantly weaker than control levels of attraction but was still attractive. Purple indicates that the mean response was not significantly different from zero, as tested by a one-sample *t*-test, and is therefore described as 'indifferent'. Blue indicates that the repulsive response was significantly different from zero. A blank rectangle indicates that the response was not significantly different from the control levels. Control responses were all tested using one-sample *t*-tests and are indicated as being significantly attractive (red), not significantly different form zero (i.e. indifferent – purple) or significantly repulsive (blue).

Increases in sensitivity following cross-adaptation have been observed in electrophysiological studies on lobster receptor cells (Borroni and Atema, 1989), rat trigeminal nerve (Farley and Silver, 1992) and the housefly antenna (Kelling et al., 2002). However, none of the data presented in Figs 4 and 5 fit this profile. In particular, although the maximal (negative) response may have been increased in some cases (e.g. Fig. 4A), there is no evidence for an increase in the

sensitivity of the olfactory system at the lower doses tested here.

This may be a function of the relatively narrow range of volumes we used, but it may also be due to the phenotype being measured. Because this study was conducted on a behavioural response, it is difficult to interpret the observed changes in response thresholds in terms of the activity of receptor neurons. The work by Kreher et al. (2005) on the response profiles of larval olfactory receptors in an adult *in vivo* expression system studied only two of the acetates used here (ethyl acetate and pentyl acetate) and casts little light on our findings. Electrophysiological studies of receptor neuron activity in both unadapted and adapted conditions and functional anatomical investigations of the organisation of central sensory structures will be necessary to prove the existence of receptor cross-talk: we are actively pursuing both these lines of research.

In a previous study (Cobb and Domain, 2000), responses to octanol were transformed from a control response of indifference to repulsion after pre-stimulation with C7–C9 alcohols. To test whether this effect may also have involved changes in attraction thresholds, the responses of larvae to varying doses of octanol were studied following pre-stimulation with octanol. Following pre-stimulation, the response to octanol was transformed into a strong repulsive response at all volumes, which was significantly different from control levels ($F_{1,96}$=63.45, P<0.0001; Fig. 7). This result further indicates that changes in response threshold underlie some examples of olfactory adaptation in *Drosophila* larvae and underlines the importance of taking into account the possibility that changes in sensitivity may underlie apparent adaptation effects. This interpretation has not been excluded in previous whole-organism studies of adaptation (e.g. Colbert and Bargmann, 1995; Cobb and Domain, 2000).

Olfactory adaptation may be of fundamental importance to the natural history of maggots. Larvae are naturally surrounded by strong odours of the kind studied here, produced by their food. Our data suggest that altered sensitivity to certain odours would naturally come about through the continued adaptation of the olfactory system to this sensory environment, enabling larvae to respond rapidly to ecologically significant changes in the chemical composition of their food, as indicated by changes in odour concentrations. This may provide an adaptive advantage to the observed induction of a repulsive response to odours that were previously attractive or induced no response.

Whatever mechanism(s) underlie these results, they provide an insight into more straightforward aspects of sensory coding, as initially intended. Methyl acetate had a major effect on all the acetic esters studied except heptyl acetate (C9) and on three alcohols and acids. In the case of the aliphatic acetates, we interpret this to mean that methyl acetate does not have a specific detection pathway but is processed by all the pathways associated with distinguishing ethyl…pentyl acetate. This interpretation is reinforced by the fact that the response to methyl acetate was not qualitatively altered by pre-stimulation with any odour: at least one pathway processing C3–C8 aliphatic acetates was open following pre-stimulation and was able to detect methyl acetate.

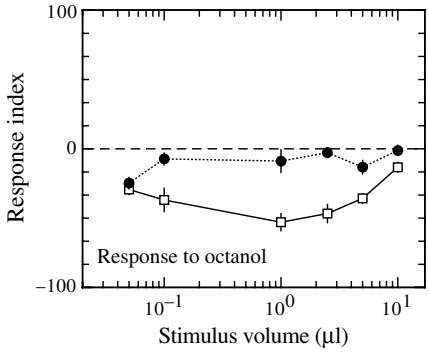

Fig. 7. Dose–response curves to test doses of octanol, showing mean response indices (N=8) and standard errors (error bars are sometimes smaller than the symbols used in the graph). Dotted line, control (no pre-stimulation); solid line, following pre-stimulation with octanol.

Similar results have been found for hexanol (Cobb and Domain, 2000), which affected responses to all other alcohols tested but was not reciprocally affected by them. That result was interpreted as an example of lateral inhibition. Here, we are less categorical, appreciating that either peripheral or central mechanisms, or both, may be involved. Our hypothesis that methyl acetate is detected by the processing pathways involved in detecting the other aliphatic acetates studied here would suggest that the cross-functional group data (Table 2), in which methyl acetate showed a major effect, may be due to the joint action of all pathways involved with processing C3–C8 acetates. One way of testing this hypothesis would be to carry out a multiple cross-adaptation test in which larvae were pre-stimulated with several or all C3–C8 acetic esters. However, it remains possible that methyl acetate has a processing pathway (either intra- or intercellular) that exerts an inhibitory effect on all others, similar to that hypothesised for hexanol.

The similar effects seen following pre-stimulation with ethyl acetate indicate that methyl and ethyl acetate share most if not all of their processing pathways. However, the striking differences in the cross-functional group data (Table 2), in which ethyl acetate had no effect on alcohols or acids, show that these odours can be distinguished by the larval olfactory neural network. The aliphatic acetate cross-adaptation data (Table 1) showed that test responses to propyl acetate were affected by pre-stimulation with all acetic esters tested here, perhaps suggesting that there is no specific propyl acetate processing pathway. The transformation of nonanoic acid into a repulsive odour following pre-stimulation with propyl acetate might disprove this hypothesis, but the effect of pre-stimulation with butyl and pentyl acetate would have to be studied first to exclude the possibility that this effect is mediated by processing pathways primarily associated with these two odours.

The data in Table 1 show that maggots respond to butyl and pentyl acetate in very similar manners, but the existence of a

specific genetic anosmia to pentyl acetate (Cobb and Dannet, 1994) shows that larvae can discriminate the two odours. Hexyl and heptyl acetate appear to be processed separately from the other odours, as shown by the lack of cross-adaptation shown by test responses to these two odours (with the exception of methyl and ethyl acetate on hexyl acetate). The existence of a specific anosmia to hexyl acetate (Cobb and Dannet, 1994) confirms this.

Taken as a whole, these data show no clear evidence for any of the 16 odours studied here being odour equivalents. Naturally occurring odour sources, to which the larval olfactory system will have been tuned by natural selection, will consist of complex mixtures of these and many other components (Stensmyr et al., 2003). Nevertheless, larvae can apparently distinguish all these odours, process them using related but separate pathways and respond to them in different manners. They achieve this with only 21 olfactory neurons and what can be assumed to be a roughly equivalent number of olfactory receptor molecule types. This neurobiological feat remains largely unexplained, but we can expect it to reveal principles of olfactory coding that may be common to a range of organisms and not merely restricted to either holometabolous larvae or even insects. The next challenge will be to discover the anatomical and biochemical nature of the pathways involved in processing these odours and how they interact to produce the olfactory response, adaptation and alterations in response threshold.

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