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Dual antennular chemosensory pathways mediate odor-associative learning and odor discrimination in the Caribbean spiny lobster *Panulirus argus*

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Summary

Chemosensory neurons in the antennular flagella of lobsters mediate long-range responses to chemicals. These neurons are part of two parallel chemosensory pathways with different peripheral and central components. Aesthetasc sensilla on the lateral flagella are innervated by chemosensory neurons that project to the olfactory lobes. A diversity of other 'non-aesthetasc' sensilla on both lateral and medial flagella are innervated by mechano- and chemosensory neurons, and most of these non-aesthetasc neurons project to the lateral antennular neuropils. We investigated the roles of these two pathways in odor-associative learning and odor discrimination by selectively removing either aesthetasc or non-aesthetasc sensilla from the spiny lobster *Panulirus argus*. Lobsters lacking both aesthetasc and non-aesthetasc antennular sensilla show very reduced or no odor-mediated searching behavior. We associatively conditioned lobsters using two paradigms: aversive conditioning with generalization testing (which reveals the similarity in the lobsters' perception of odorants) and discrimination conditioning (which reveals the lobsters' ability to discriminate odorants). Sham-control intact lobsters performed these tasks well, as did lobsters lacking either aesthetascs or non-aesthetasc setae. There was a strong but statistically non-significant trend that lobsters lacking either aesthetascs or non-aesthetasc setae generalized more between complex odor mixtures than did intact lobsters. After aversive conditioning with generalization testing, aesthetasc-ablated lobsters had more difficulty discriminating among the most closely related complex mixtures than did intact or non-aesthetasc-ablated lobsters. However, after discrimination conditioning, aesthetasc-ablated lobsters were as proficient as intact animals in discriminating highly similar mixtures. These results indicate overlap and redundancy in the function of these two chemosensory pathways in odor-associative learning and odor discrimination, but these pathways also complement each other to enable better discrimination. This study presents the first evidence for a role of non-aesthetasc chemosensory neurons in complex odor-mediated behaviors such as learning and discrimination.

Key words: Crustacea, olfaction, chemoreception, chemical sense, odour discrimination, odour-associative learning, aesthetasc, sensory, olfactory lobe, lateral antennular neuropil, Caribbean spiny lobster, *Panulirus argus*.

Introduction

Olfactory systems from phylogenetically diverse animals have similar organizational features. Afferents of olfactory receptor neurons terminate in glomeruli in primary olfactory centers in the brain of vertebrates (Pinching and Powell, 1971; Mori and Yoshihara, 1995; Hildebrand and Shepherd, 1997; Mori et al., 1999) and many invertebrates; for example, molluscs (Chase and Tolloczko, 1993), insects (Maynard, 1966; Tolbert and Hildebrand, 1981; Boeckh et al., 1984; Laissue al., 1999) and crustaceans (Mellon and Munger, 1990; Sandeman et al., 1992; Schmidt and Ache, 1996b). Odor quality is represented in glomerular neuropils as spatial odotopic patterns (Cinelli et al., 1995; Mombaerts et al., 1996; Friedrich and Korsching, 1998; Galizia et al., 1999; Sachse et al., 1999; Wachowiak et al., 2000; Belluscio and Katz, 2001). The fact that olfactory glomerular neuropils may have evolved independently among several arthropod groups (Strausfeld, 1998) and in other phyla suggests that a glomerular organization to the primary olfactory neuropils might be a superior adaptation to minimize neuropilar volume and to capture the diverse and complex nature of chemical signals (Hildebrand and Shepherd, 1997).

The olfactory neurons of some invertebrates, such as snails (Chase and Tolloczko, 1993) and decapod crustaceans (Schmidt and Ache, 1996a), project into non-glomerular neuropils, suggesting that non-glomerular neuropilar organizations can also efficiently process chemosensory information. The glomerular and non-glomerular pathways operate in parallel in some species. For example, in lobsters and other decapods,

chemosensory neurons in the flagella of the first antennae, or antennules (Fig. 1), which mediate long-range responses to chemicals (McLeese, 1973; Reeder and Ache, 1980; Devine and Atema, 1982; Giri and Dunham, 1999, 2000; Steullet et al., 2001), project into two paired neuropils of the brain: the olfactory lobes (OLs) and lateral antennular neuropils (LANs) (Sandeman and Denburg, 1976; Schmidt et al., 1992; Schmidt and Ache, 1992, 1996a,b; Helluy et al., 1996). The OLs contain many glomeruli (e.g. 1000 in the spiny lobster *Panulirus argus*) and receive input primarily from chemosensory neurons associated with aesthetasc sensilla (Mellon and Munger, 1990; Schmidt and Ache, 1992, 1996b; Sandeman and Sandeman, 1994). These unimodal sensilla are located exclusively on the distal part of the antennular lateral flagella (Fig. 1), and each is innervated by several hundred chemosensory neurons (Spencer and Linberg, 1986; Grünert and Ache, 1988; Steullet et al., 2000). Each aesthetasc contains similar populations of chemosensory neuron types, and thus each appears to be a functional unit of odor quality coding (Steullet et al., 2000).

In contrast, the LANs are multimodal sensory-motor processing centers. They receive input from non-aesthetasc sensilla, which are diverse types of bimodal (chemo- and

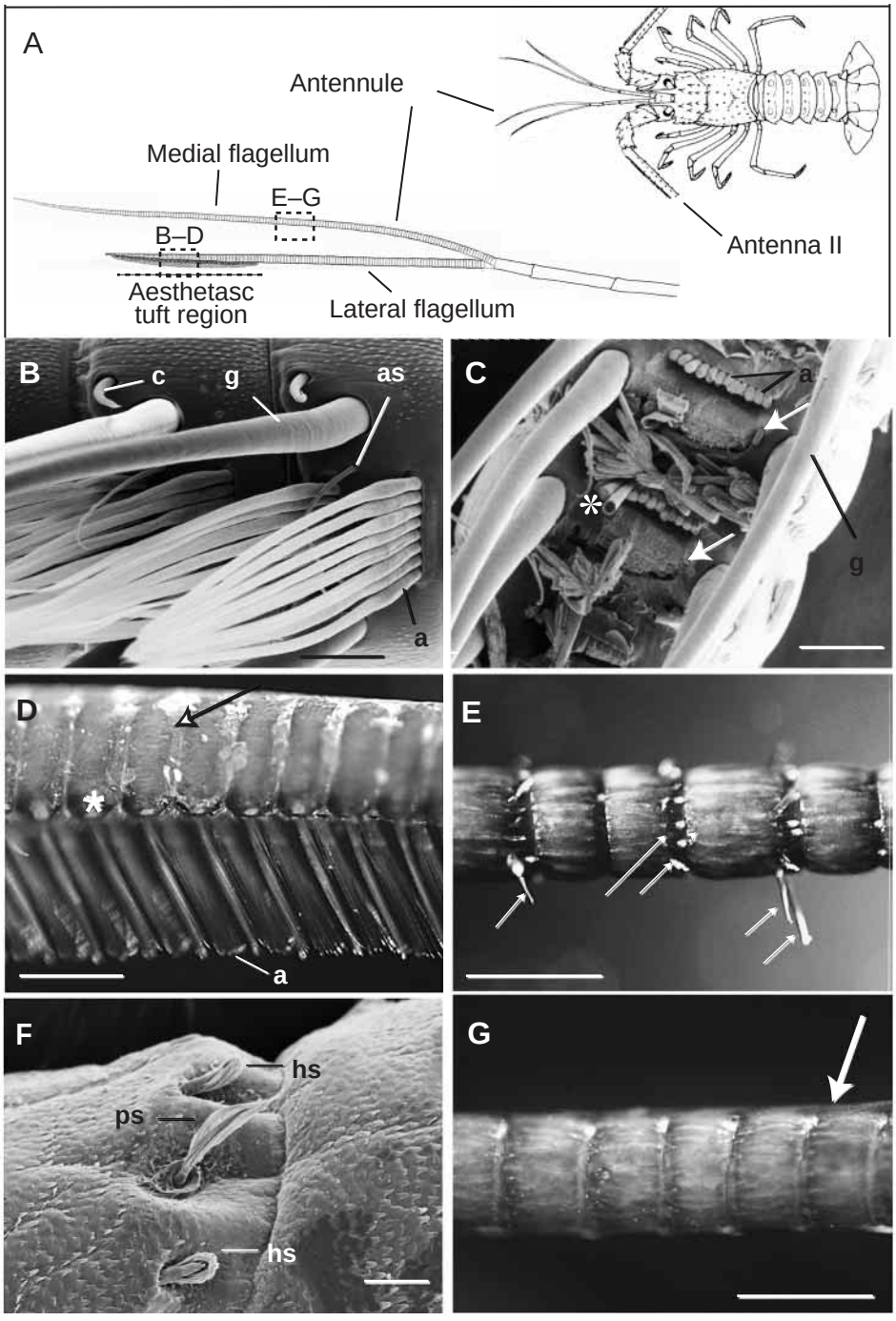

Fig. 1. Intact and ablated antennular medial and lateral flagella of the spiny lobster. (A) Drawing of a lobster showing the antennules (first antennae) and antennae II (second antennae). The higher-magnification drawing of an antennule shows the medial and lateral flagella. Aesthetascs are located exclusively on the distal half of the lateral flagellum, in the aesthetasc tuft region. Non-aesthetasc chemosensilla are located along the entire length of both the lateral and medial flagella. Letters B–G indicate the position on the antennule where respective micrographs were taken. (B) Scanning electron micrograph of the aesthetasc region of an intact lateral flagellum with rows of aesthetascs (a) and accompanying setae: companion setae (c), guard setae (g) and asymmetric setae (as). (C) Scanning electron micrograph of the aesthetasc tuft region after shaving aesthetascs (a) and asymmetric setae (arrows), but not other setae including guard setae (g). The asterisk marks an aesthetasc whose base was not completely removed by shaving (in these aesthetasc bases, dendrites were also completely disrupted, as shown by histological techniques; data not shown). (D) Light micrograph of the aesthetasc tuft region covered by cyanoacrylate glue (arrow) after shaving all setae except aesthetascs (a) and asymmetric setae (not visible on this micrograph). The asterisk indicates the original location of a guard seta prior to shaving. (E) Light micrograph of a region of an intact medial flagellum. Arrows indicate setae. (F) Scanning electron micrograph of a region of an intact medial flagellum showing two types of non-aesthetasc sensilla: hooded sensillum (hs) and plumose seta (ps). Hooded sensilla house both mechano- and chemosensory neurons [modified from (Cate and Derby, 2001)]. (G) Light micrograph of a region of a medial flagellum covered by cyanoacrylate glue (arrow) after shaving all setae. Scale bars: B, 100 μm; C, 150 μm; F, 50 μm; D,E,G, 400 μm.

mechano-) sensilla on the lateral and medial flagella (Fig. 1) (Cate and Derby, 2000, 2001, 2002a,b). The LANs also contain arborizations of antennular motoneurons (Schmidt et al., 1992; Schmidt and Ache, 1993, 1996a). On the basis of the organization of the OLs and LANs, it has been postulated that the LANs, and consequently non-aesthetasc chemosensory neurons, are involved in antennular movements and other simple and reflexive behaviors driven by chemo- and mechanosensory inputs from the antennules, whereas the OLs, and hence aesthetasc chemosensory neurons, are implicated in more complex odor-mediated behaviors such as discrimination and orientation (Maynard, 1966; Schmidt and Ache, 1993).

The aim of our study was to use the Caribbean spiny lobster *Panulirus argus* to investigate the functional role of both aesthetasc and non-aesthetasc chemosensory neurons of the antennules, and hence of both antennular chemosensory pathways, in odor-associative learning and odor discrimination. The role of these two distinct pathways in odor-mediated activation of searching and orientation is described elsewhere (Horner et al., 2000; Derby et al., 2001; Steullet et al., 2001). In the present study, we show, by making specific ablations of either aesthetasc or non-aesthetasc sensilla on the antennules, that either the aesthetasc or the non-aesthetasc pathway is sufficient but not necessary to mediate odor-associative learning and odor discrimination.

Materials and methods

Animals

Lobsters *Panulirus argus* (Latreille, 1804) (55–75 mm carapace) were collected in the Florida Keys, shipped to Georgia State University, kept under a 12 h:12 h light:dark photoperiod in 800 l aquaria containing aerated, recirculated, filtered artificial sea water (ASW) (Instant Ocean, Aquarium Systems, Mentor, OH, USA) and fed squid or shrimp. Intermolt lobsters, determined by the method of Lyle and MacDonald (1983), were selected for behavioral assays if they responded to a piece of squid or shrimp introduced into the tank. Selected lobsters were placed individually in 80 l aquaria (60 cm long × 30 cm wide × 45 cm high) containing filtered ASW, which was vigorously mixed and aerated using a recirculating pump, and a layer of crushed coral gravel covering the bottom. The lobsters were acclimated to their new environment under a 12 h:12 h light:dark photoperiod for several days prior to testing. During the acclimation period and pre-conditioning testing, lobsters were fed approximately 5 g of squid every 2–3 days.

Odorants

Oyster extract (OE) was prepared as described by Carr and Derby (1986), stored in samples at –80 °C and diluted to 30 g tissue l^{-1} ASW prior to use. The compositions of artificial oyster odor (OO), crab odor (CO), inverse crab odor (ICO), shrimp odor (SO) and mullet odor (MO) are given in Table 1 (Carr and Derby, 1986). All odorants were prepared in ASW. OO was used in the electrophysiological evaluation of the

Table 1. *Composition of the complex odors used in this study*

	Concentration (μmol l^{-1}) of components in complex odors				
Component	CO	ICO	SO	MO	OO
Alanine	50.4	2.8	63.2	32.2	90.4
β-Alanine	0	0	0	0.3	21.2
α-Aminobutyrate	0	0	0	0.4	0
Arginine	53.8	2.5	26.2	3.4	7.6
Asparagine	4.6	7.5	3.0	4.1	2.8
Aspartate	0.8	102.4	2.5	4.1	12.1
Cysteine	2.8	50.4	0	0	1.6
Glutamate	6.7	5.3	4.9	5.1	12.5
Glutamine	69.4	2.4	18.8	6.2	9.6
Glycine	266	0.6	278	37.4	59
Histidine	3.2	20.6	1.0	82	1.2
Hydroxyproline	3.8	9.5	0	0.8	3.0
Isoleucine	2.5	53.8	3.1	1.8	0.4
Leucine	5.8	6.2	5.7	3.1	1.0
Lysine	5.3	6.7	1.4	14.3	4.1
Methionine	9.2	4.3	3.1	0.9	0.3
3-Methylhistidine	0	0	0	1.5	0
Ornithine	0	0	0	3.3	2.3
Phenylalanine	2.2	79.6	1.6	1.1	0.1
o-Phosphoserine	0	0	0	1.1	0
Proline	131	0.8	34	5.7	30.4
Serine	6.2	5.8	4	6	8.3
Taurine	44.2	3.1	98.2	140	408
Threonine	9.5	3.8	1.9	5.9	1.4
Tryptophan	3.1	44.2	0	0	0
Tyrosine	2.4	69.4	2.7	0.4	0.6
Valine	7.5	4.6	6.8	3.3	1.0
Adenosine 5′-monophosphate	0.6	266	14.5	0.2	7.3
Adenosine 5′-diphosphate	4.3	9.2	4.2	0.1	0.6
Adenosine 5′-triphosphate	20.6	3.2	1.2	0	0
Guanosine 5′-monophosphate	0	0	0	0.7	1.0
Inosine 5′-monophosphate	3.7	11.0	5.9	50.4	1.4
Xanthosine 5′-monophosphate	0	0	0	2.1	0
Hypoxanthine	0.8	131	0.7	0.9	0
Inosine	1.6	87	0.2	12.1	0.9
Betaine	87	1.6	150	76	250
Homarine	11.0	3.7	20.6	0	24.6
Trimethylamine oxide	79.6	2.2	160.8	72.4	0
L-Lactate	102.4	0.8	81.8	420	6.7
D-Lactate	0	0	0	0	1.1
Succinate	0	0	0	0	26.4

Each complex odor has a total concentration of 1 mmol l^{-1}. All amino acids are the L-isomer.

CO, crab odor; ICO, inverse crab odor; SO, shrimp odor; MO, mullet odor; OO, oyster odor. These recipes are from Carr and Derby (1986).

ablations; CO, ICO, SO and MO were used in behavioral tests. Blend ratios of the binary mixture adenosine 5′-monophosphate (AMP) and taurine were also used at a total concentration of 1 mmol l^{-1}. The compositions of the blend ratios were 0.999 mmol l^{-1} AMP + 0.001 mmol l^{-1} taurine (99.9:0.1), 0.99 mmol l^{-1} AMP + 0.01 mmol l^{-1} taurine (99:1), 0.90 mmol l^{-1} AMP + 0.10 mmol l^{-1} taurine (90:10) and 0.50 mmol l^{-1} AMP + 0.50 mmol l^{-1} taurine (50:50). The purity of all chemicals was >99 %.

Ablations

To perform ablations and sham ablations, odorant-responsive lobsters were immobilized in an ASW-filled container. The following bilateral antennular ablations were performed.

Chemoreceptor-ablated animals

Exposure to distilled water functionally ablates chemosensory neurons in lobsters and other marine crustaceans by osmotically disrupting the outer dendrites of chemosensory neurons that are located in the permeable chemosensilla (Derby and Atema, 1982; Gleeson et al., 1996, 2000). Distilled water ablation was accomplished by placing all antennular flagella in 15 ml centrifuge tubes containing distilled water for 15–30 min.

Sham-control animals

These animals were immobilized in the ASW tray for approximately 30 min, and antennular flagella were placed in centrifuge tubes containing ASW for 15–30 min.

Aesthetasc-ablated animals

Physical removal of setae eliminated the activity of their chemoreceptor neurons, for two reasons: (i) shaving removes the chemoreceptor neurons' receptor sites, which are located on the neurons' dendrites in the setal shafts (Spencer and Linberg, 1986; Grünert and Ache, 1988; Blaustein et al., 1993; Gleeson et al., 1996, 2000; Cate and Derby, 2001, 2002a); and (ii) shaving causes rapid death and degeneration of neurons innervating the shaved sensilla (Harrison et al., 2001). Shaving of aesthetasc sensilla was performed under a compound microscope using a 0.2 mm wide piece of carbon steel blade, which allowed removal of aesthetascs without damaging the neighboring guard and companion setae. Shaving aesthetascs removes the outer dendrites of the chemosensory neurons and completely disrupts the inner dendrites within any remaining bases of shaved sensilla. This ablation also removes the asymmetric setae, which are located just lateral to aesthetascs. The innervation of asymmetric setae is unknown, but they may contain chemosensory neurons that project to the OLs (Schmidt and Ache, 1996a).

Non-aesthetasc-ablated animals

Ablation of non-aesthetasc chemosensory neurons was accomplished by shaving all visible setae on both medial and lateral flagella, with the exception of aesthetascs and asymmetric setae, and by subsequently covering the flagella (again, except for aesthetascs and asymmetric setae) with a uniform layer of cyanoacrylate glue. Because covering appendages with cyanoacrylate glue efficiently prevents chemical responses (Derby and Atema, 1982), we used this method as a supplementary measure to cover any remaining small or unshaven setae. This treatment also resulted in functional disruption of most antennular mechanosensory neurons, since many of the non-aesthetasc setae contain mechanoreceptor neurons in addition to chemoreceptor neurons (Cate and Derby, 2000, 2001, 2002a,b). The distal tip of each lateral flagellum, which consists of small annuli without aesthetascs, was also removed. During this procedure, aesthetascs were carefully maintained in an ASW-filled groove to prevent desiccation and physical damage.

Evaluation of efficacy of ablations

Morphology

Following completion of the behavioral assays, flagella of lobsters on which ablations had been performed were cut and fixed in 2.5 % glutaraldehyde, 1 % paraformaldehyde, 10 % sucrose in 0.2 mol l^{-1} phosphate buffer solution at pH 7.4. The quality of ablation was evaluated under high magnification using a Zeiss Axioskop compound microscope.

Electrophysiology

The efficacy of ablations was also evaluated by quantifying odorant-evoked electrical activity from the axons of chemoreceptor neurons. The electrophysiological preparation used is described in detail in Derby (1995). A continuous stream of ASW flowed at 10 ml min^{-1} over the flagellum. Stimuli were delivered by using an electronically driven valve to insert a 3 s pulse of stimulus at the same flow rate. Interstimulus intervals were 1 min. For a flagellum, electrical activity was recorded using a suction electrode positioned randomly at 20 different sites along different axonal bundles of the antennular nerve. The electrode tip was large enough to record multiple units simultaneously. For each recording site, responses to ASW and artificial oyster odor (OO) at 1 mmol l^{-1} total concentration were recorded. Extracellular recordings were digitized using Axoscope 8.0 (Axon Instruments Inc.) and analyzed using Data-Pac III (Run Technology). For each recording, electrical activity was quantified as the number of action potentials that exceeded a threshold level set just above the upper limit of the electrical noise of the recording before odor stimulation. Electrical activity generated by an odorant was quantified as the number of action potentials occurring in the first 1 s of the response following stimulation minus the number of action potentials occurring during the 1 s period before stimulation. Finally, the odorant-evoked activity of an antennule was quantified by summing the activity of all 20 independent recording sites on the nerve bundles of that flagellum.

Behavioral assays

All behavioral assays were conducted in the 80 l aquaria described above under a dim red light during the dark phase of the 12 h:12 h light:dark cycle.

Role of antennular chemosensory neurons in odorant-mediated search behavior

The aim of this experiment was to assess the effect of distilled-water ablation of chemosensory neurons in the antennular medial and lateral flagella on search responses to CO.

Pre-ablation test. ASW and three concentrations of CO (0.05, 0.5 and 5 mmol l^{-1}) were presented to lobsters in a random order by an observer unaware of the identity of each stimulus. Each stimulus was presented twice per day, with at least 15 min between stimulations, for two consecutive days. Stimuli consisted of 5 ml solutions delivered to the antennules within 2 s from a hand-held glass pipette whose tip was carefully positioned approximately 5 cm from the antennules of an inactive lobster. After reaching the antennules, the stimulus was rapidly dispersed and diluted by the vigorous flow in the aquarium. Responses were quantified by measuring the duration of 'search' behavior during 3 min following stimulation. A search response was defined as forward or lateral movement of a lobster. From the 2 days of testing, the mean response to ASW was subtracted from the mean response to each odorant concentration. Lobsters that did not respond to odorants or responded only weakly in a concentration-independent manner during the pre-ablation phase (five of 28 animals) were removed from the study.

Ablation. For the treated lobsters, both antennular lateral and medial flagella were treated with distilled water as described above. For control animals (sham ablation), the antennules were placed in ASW rather than distilled water.

Post-ablation test. One day after ablation or sham ablation, lobsters were tested twice with ASW and the three concentrations of CO (0.05, 0.5 and 5 mmol l^{-1}) as in the pre-ablation test. Search responses were corrected for ASW responses as described in the pre-ablation test. Lobsters that searched in response to ASW for longer than 42 s (corresponding to the 99th percentile of all ASW responses of all animals in all experiments) during either the pre- or post-ablation phase were discarded *post-hoc*; this excluded the nine most unpredictable animals that tended to be highly active independent of odorant stimulation. For both the distilled-water-treated lobsters and the sham control lobsters, post-ablation responses were compared with pre-ablation responses using one-way within-subjects analysis of variance (ANOVA) with multiple dependent measures (MANOVA).

Role of aesthetascs and non-aesthetasc setae in olfactory learning and discrimination

The aim of this experiment was to determine the roles of aesthetascs and non-aesthetasc setae in odor learning and discrimination. Discrimination was evaluated in intact, aesthetasc-ablated and non-aesthetasc-ablated lobsters using a conditioning paradigm consisting of three phases: (i) a pre-conditioning phase, during which lobsters were tested for responsiveness to oyster extract (OE, see *Odorants*); (ii) a conditioning phase, during which an odorant (the conditioned stimulus, CS+) was forward-paired with an aversive unconditioned stimulus (US); and (iii) a post-conditioning phase, during which lobsters were stimulated randomly and blindly with the CS+ and other non-conditioned but related odorants. To assess the effect of conditioning in each group of lobsters, the responses of conditioned and unconditioned animals were compared.

Odorants were presented by delivering 5 ml of stimulus within 2 s from a hand-held glass pipette whose tip was positioned approximately 5 cm away from the antennules of an inactive lobster. The US was a black plastic panel (6.5 cm×9.5 cm) mounted on a rod that was rapidly moved towards the lobster until the animal walked or tail-flipped away. The US was presented 3 s after the delivery of the CS+.

Two slightly different conditioning paradigms were used: (i) aversive conditioning with odor generalization testing; and (ii) discrimination conditioning. Both paradigms are described in detail by Livermore et al. (1997), and they are briefly presented in the following section.

Aversive conditioning with odor generalization testing

This paradigm (Table 2) tested how lobsters generalize between complex odor mixtures with related but different compositions – specifically, between an aversively conditioned complex mixture (CS+) and three novel, non-conditioned complex mixtures.

Pre-conditioning phase. Pre-conditioning tests started the day after ablation or sham control treatment. This consisted of delivering OE (30 g tissue l^{-1} ASW) and ASW to the antennules of an inactive lobster twice a day with at least 15 min between stimulations, using a random and blind procedure. Responses were quantified by measuring the duration of search behavior during 3 min following odorant stimulation as described in the previous section. The response to ASW was subtracted from the response to OE. Pre-conditioning tests were repeated every day until lobsters responded consistently to OE for at least two consecutive days. Lobsters that did not consistently respond to OE within approximately a week following ablation or sham control treatment (19 of 94 animals) were discarded.

Conditioning phase. CO at 1 mmol l^{-1} total concentration was the CS+ and was forward-paired with the US. Such forward pairing of the CS+ and US was repeated three times per day for two consecutive days. During this conditioning phase, ASW was also presented three times randomly between the conditioning trials to ensure that learning was associated with features of the CS+ and not simply with the mechanical component of stimulus presentation. The interval between forward pairings or ASW presentations was 15 min.

Post-conditioning phase. The CS+ and the novel non-conditioned odors (ICO, SO, MO), all at 1 mmol l^{-1} total concentration, and ASW were presented randomly and blindly twice a day with at least 15 min between stimulations for two consecutive days. The search response to each stimulus was quantified as in the pre-conditioning phase. Responses to these mixtures (after subtraction of the ASW response) were standardized to the mean response to OE (after subtraction of the ASW response) during the last 2 days of the pre-conditioning phase.

Table 2. *Aversive conditioning with generalization testing to assess the ability of lobsters to generalize and discriminate between an aversively conditioned complex odor (CS+=CO) and other novel, non-conditioned complex odors (ICO, SO and MO)*

	Pre-conditioning	Conditioning	Post-conditioning
Lobsters aversively conditioned to crab odor (CO)			
Stimuli	OE	CO forward-paired with aversive stimulus	CO (=CS+) ICO SO MO
	ASW	ASW	ASW
Stimulation protocol	Twice per day For at least 2 days	Three times per day For 2 consecutive days	Twice per day For 2 consecutive days
Unconditioned lobsters			
Stimuli	OE		CO ICO SO MO
	ASW		ASW
Stimulation protocol	Twice per day For at least 2 days		Twice per day For 2 consecutive days

OE, oyster extract; CO, crab odor; ICO, inverse crab odor; SO, shrimp odor; MO, mullet odor; ASW, artificial sea water.

Discrimination conditioning

This paradigm (Table 3) assessed the ability of lobsters to discriminate among odorants. As in the previous paradigm, one odorant (CS+) was aversively conditioned by forward-pairing it with the aversive US during the conditioning phase. But, unlike in the previous paradigm, other odorants were presented explicitly unpaired with the US during the conditioning phase. Thus, these other non-conditioned odorants became the conditioned inhibitor of the aversive stimulus (i.e. CS–) and served as a safety signal indicating that the aversive stimulus would not occur (Rescorla, 1969; Mackintosh, 1973; Livermore et al., 1997). During the conditioning phase, the CS– odorants and ASW were delivered twice randomly between the three forward pairings of the CS+ and US. The interval between stimulations was at least 15 min. Search responses during the pre- and post-conditioning phases were quantified as in the generalization paradigm described above, but only during the first 1 min following stimulation. After the pre-conditioning phase, 33 of 97 lobsters were discarded because of their lack of consistent responses to OE.

The odorants used in this paradigm were blend ratios of the binary mixture of AMP and taurine, each blend at a total concentration of 1 mmol l^{-1}. We used these blend ratios rather than the complex mixtures used in the other protocol because we wanted to make the discrimination task more difficult. The results of the previous procedure indicated that ablations had little effect on learning and discrimination. Therefore, to increase the likelihood of observing the effects of ablation, we selected stimuli that were even more similar and therefore should be more difficult for lobsters to discriminate. The CS+ was blend ratio 99.9:0.1 (which is 0.999 mmol l^{-1} AMP and 0.001 mmol l^{-1} taurine). CS– blend ratios of AMP and taurine were 99:1, 90:10 and 50:50. Taurine and AMP were chosen because both odorants stimulate antennular chemosensory neurons (Derby et al., 1991; Cromarty and Derby, 1997; Steullet and Derby, 1997) and behavior (Lynn et al., 1994; Livermore et al., 1997; Derby, 2000) and they elicit similar levels of search responses when presented alone at 1 mmol l^{-1} (data not shown). Because responses to binary mixtures can be low, lobsters were stimulated twice with OE at the end of the post-conditioning phase to ensure that weak responses to blend ratios during the post-conditioning phase were not due to loss of olfactory responsiveness. Lobsters that did not respond to OE at the end of post-conditioning phase were considered unresponsive to odorants and were discarded *post-hoc*. Only one of the 64 lobsters was removed from the analysis.

Results

Efficacy of ablations

Morphological evaluation

Ablation of aesthetascs resulted in the removal of 99.9±0.02 % of aesthetascs (mean ± S.E.M., N=48), and thus aesthetasc-ablated lobsters possessed, on average, only 2.5 intact aesthetascs. An example is shown in Fig. 1C. Further evidence that the removal of aesthetascs eliminates the function of aesthetasc chemoreception is the fact that shaving causes the chemosensory neurons and the glial cells associated with the aesthetasc to degenerate (Harrison et al., 2001).

Table 3. *Discrimination conditioning to assess the ability of lobsters to discriminate between an aversively conditioned blend ratio of a binary mixture of AMP+taurine (CS+=99.9:0.1) and other 'safe' conditioned blend ratios of the same binary mixture (CS−=99:1, 90:10 and 50:50)*

	Pre-conditioning	Conditioning	Post-conditioning
Lobsters aversively conditioned to AMP+taurine at blend ratio of 99.9:0.1			
Stimuli	OE	99.9:0.1 forward paired with the aversive stimulus	99.9:0.1 (=CS+)
		99:1	99:1 (=CS−)
		90:10	90:10 (=CS−)
		50:50	50:50 (=CS−)
	ASW	ASW	ASW
			OE*
Stimulation protocol	Twice per day	Three times per day for forward pairing and ASW	Twice per day
		Twice per day for other three blend ratios	
	For at least 2 days	For 2 consecutive days	For 2 consecutive days
Unconditioned lobsters			
Stimuli	OE		99.9:0.1
			99:1
			90:10
			50:50
	ASW		ASW
Stimulation protocol	Twice per day		Twice per day
	For at least 2 days		For 2 consecutive days

*Oyster extract (OE) was tested twice after the post-conditioning phase to ensure that any weak responses to CS+ and CS− during the post-conditioning phase were not due to loss of odor responsiveness.

ASW, artificial sea water.

Similarly, the quality of ablation of non-aesthetasc chemosensory neurons was evaluated by counting the number of visible intact setae that were not covered by glue on both lateral and medial flagella of non-aesthetasc-ablated lobsters. Examples are shown for non-aesthetasc ablation of the antennular lateral flagellum (Fig. 1D) and medial flagellum (Fig. 1E,G). Overall, only 35±5 non-aesthetasc setae (mean ± S.E.M., N=14) were found intact and uncovered by glue on non-aesthetasc-ablated lobsters. This corresponded to an approximately 99.4 % reduction of non-aesthetasc setae on the antennules of non-aesthetasc-ablated lobsters. Moreover, 70±4.5 % of aesthetascs (mean ± S.E.M., N=14) remained undamaged by the end of the experiments on non-aesthetasc-ablated animals. Damaged aesthetascs were located primarily on the most distal aesthetasc-bearing annuli. This damage occurred because aesthetascs were not protected by guard setae, and these animals performed more grooming of their antennules than normal.

Electrophysiological evaluation

Removal of either aesthetascs or non-aesthetasc setae decreased the odorant-evoked activity in the nerves of lateral flagella (Fig. 2). The greatest reduction occurred after removal of aesthetascs, which house the largest proportion of chemosensory neurons on the lateral flagella (Cate and Derby, 2001). Thus, the odorant-evoked activity was significantly reduced after ablation of the aesthetascs only (planned-comparisons one-way ANOVA, P<0.05, Fig. 2), and was slightly but not significantly decreased after ablation of the non-aesthetasc setae only (planned-comparisons one-way ANOVA, P>0.05, Fig. 2). After 'total ablation' (i.e. ablation of non-aesthetasc setae and the subsequent shaving of all aesthetascs and asymmetric setae), the odorant-evoked activity was significantly smaller than after ablation of aesthetascs only (planned-comparisons one-way ANOVA, P<0.05) and was close to zero (Fig. 2). This suggests that ablation of either aesthetascs or non-aesthetasc setae on lateral flagella was largely or completely effective in removing the intended chemoreceptor neurons. Moreover, ablation of non-aesthetasc setae on medial flagella completely and significantly eliminated odorant-evoked activity in medial flagella (one-way ANOVA, P<0.05, Fig. 2).

Effects of ablation of antennular flagellar chemosensory neurons on odor-activated search behavior

Distilled-water treatment of all four antennular flagella

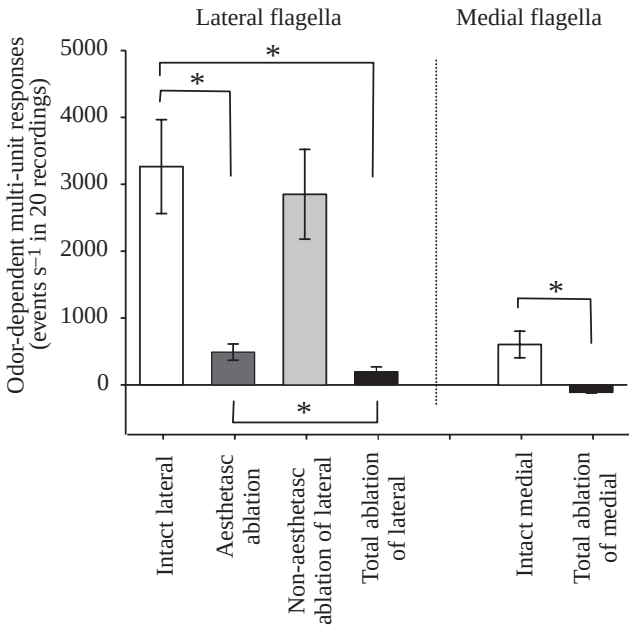

Fig. 2. Effect of ablations of aesthetascs and non-aesthetasc setae on odor-evoked responses recorded from nerves of antennular lateral and medial flagella. Six conditions are shown: (i) intact lateral (N=6), (ii) aesthetasc ablation (N=4); (iii) non-aesthetasc ablation of lateral (N=4), (iv) total ablation of lateral (N=5), (v) intact medial (N=4) and (vi) total ablation of medial (N=3). For more details, see Materials and methods. Values are means ± S.E.M. *Odor-evoked responses are significantly different from each other (planned-comparisons one-way ANOVA, P<0.05). For the planned comparisons, critical values for a 5% experiment-wise error rate were determined by the sequential Bonferroni test using the Dunn–Šidák method (Sokal and Rohlf, 1998).

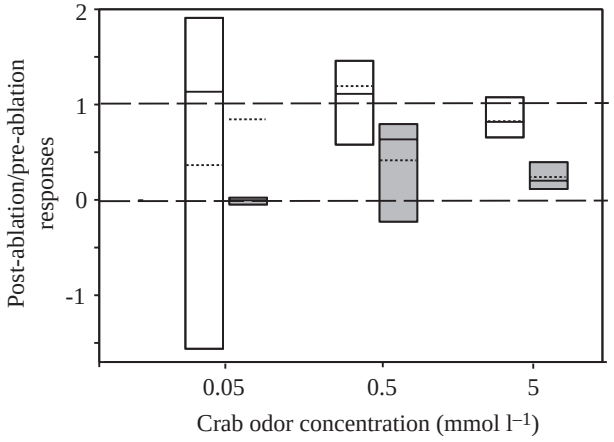

Fig. 3. Effect of distilled-water treatment of antennular flagellar chemosensory neurons on search responses to a concentration series of artificial crab odor. The ordinate represents the ratio between the post-ablation and pre-ablation responses. A value of one indicates no effect of ablation treatment, and a value of zero indicates complete elimination of responses after ablation. Each column shows the upper and lower quartiles with the median (solid line) and the mean (dotted line). N=7 lobsters in each group. Distilled-water treatment of all antennular flagella significantly reduced search responses (shaded columns) [one-way within-subjects ANOVA with multiple dependent measures (MANOVA), P=0.02]. Sham control lobsters (open columns) were not affected (MANOVA, P=0.62). For further details, see Materials and methods.

significantly reduced search responses to CO [one-way within-subject ANOVA with multiple dependent measures (MANOVA), P<0.05, Fig. 3]. Responses to 0.5 and 5 mmol l^{-1} CO were reduced by approximately 50% and 80% respectively. Responses to 0.05 mmol l^{-1} CO prior to any ablation treatment were very weak, which caused the percentage change in search response following ablation to be highly variable and thus not very informative. Reductions in search responses to CO were not observed for sham control lobsters (MANOVA, P>0.05, Fig. 3). These results show that antennular flagella primarily mediate odor-activated searching, which is the behavior used as the dependent measure in our subsequent analyses of learning and discrimination. The fact that some odorant-evoked search responses, although weak, still occurred after removal of chemoreceptors on antennular flagella suggests that chemosensory neurons located elsewhere can also mediate this behavior, although much less effectively.

Odor learning and generalization among complex odor mixtures

The aversive conditioning with generalization testing protocol (Table 2, using CO as the CS+ odor, and ICO, SO and MO as non-conditioned odors) is especially useful in evaluating the ability of animals to assess perceptual similarities between conditioned and novel odors. The results from this paradigm are shown in Figs 4 and 5.

Intact lobsters

The results in Fig. 4A show that intact lobsters learned to avoid the conditioned odor CO and could discriminate CO from the novel odors SO, MO and ICO to a lesser extent. Intact lobsters did not generalize between the conditioned odor CO and the novel odors. Unconditioned intact (sham control) lobsters did not respond differently to CO, ICO, SO or MO (one-way ANOVA with odors as repeated measures, $F_{3,56}$=0.459, P=0.712) (Fig. 4A). They searched for 29±2 s (mean ± S.E.M., N=60) following stimulation with these odors. Intact lobsters conditioned to avoid CO responded less to CO than did unconditioned intact lobsters, whereas conditioned and unconditioned intact lobsters responded equally well to the non-conditioned odors ICO, SO and MO (Fig. 4A). A two-way ANOVA (conditioning treatment as independent variable, odor type as dependent variable) revealed a significant odor effect ($F_{3,78}$=3.966, P=0.011) and a significant interaction effect between odor type and conditioning treatment ($F_{3,78}$=6.342, P=0.0007). Only responses to CO were significantly smaller in conditioned animals compared with unconditioned animals (planned-comparisons one-way ANOVA, P<0.05, Fig. 4A). Furthermore, for conditioned intact lobsters, responses to CO

were significantly smaller than to the novel complex odors SO and MO (planned-comparisons one-way ANOVA, $P<0.05$, Fig. 4A). The difference between responses to the conditioned CO and to ICO was also close of being statistically significant (planned-comparisons one-way ANOVA, $0.05<P<0.10$, Fig. 4A).

Aesthetasc-ablated lobsters

The results presented in Fig. 4B show that aesthetasc-ablated lobsters learned to avoid the conditioned odor CO, could discriminate CO from SO, but generalized to some extent between CO and the non-conditioned odors, particularly ICO. Unconditioned aesthetasc-ablated lobsters did not respond differently to the four complex odor mixtures (one-way ANOVA with odors as repeated measures, $F_{3,48}=0.600$, $P=0.618$) (Fig. 4B). They searched for 36±1 s (mean ± S.E.M., $N=52$) following odor stimulation. Aesthetasc-ablated lobsters that were conditioned to avoid CO responded less to CO and the non-conditioned odors than did unconditioned aesthetasc-ablated lobsters (Fig. 4B). A two-way ANOVA (conditioning treatment as independent variable, odor type as dependent variable) revealed a significant conditioning effect ($F_{1,23}=15.308$, $P=0.0007$) and a significant odor effect ($F_{3,69}=4.254$, $P=0.0081$). There was also a non-significant trend towards an interaction effect between odor type and conditioning treatment ($F_{3,69}=2.435$, $P=0.0721$). Responses to CO were significantly smaller in conditioned than in unconditioned aesthetasc-ablated lobsters (planned-comparisons one-way ANOVA, $P<0.05$, Fig. 4B). This was also true for the non-conditioned odors ICO and MO (planned-comparisons one-way ANOVA, $P<0.05$, Fig. 4B). Responses of conditioned aesthetasc-ablated lobsters to CO were significantly smaller than those to SO (planned-comparisons one-way ANOVA, $P<0.05$), but not to ICO and MO (planned-comparisons one-way ANOVA, $P>0.05$, Fig. 4B). The ability of aesthetasc-ablated lobsters to learn the odor-associative task and to discriminate partially between complex odors was not due to animals with incomplete ablations because aesthetasc-ablated lobsters with no remaining aesthetascs did not behave differently from those with a few intact aesthetascs (three-way ANOVA with conditioning treatment and the presence

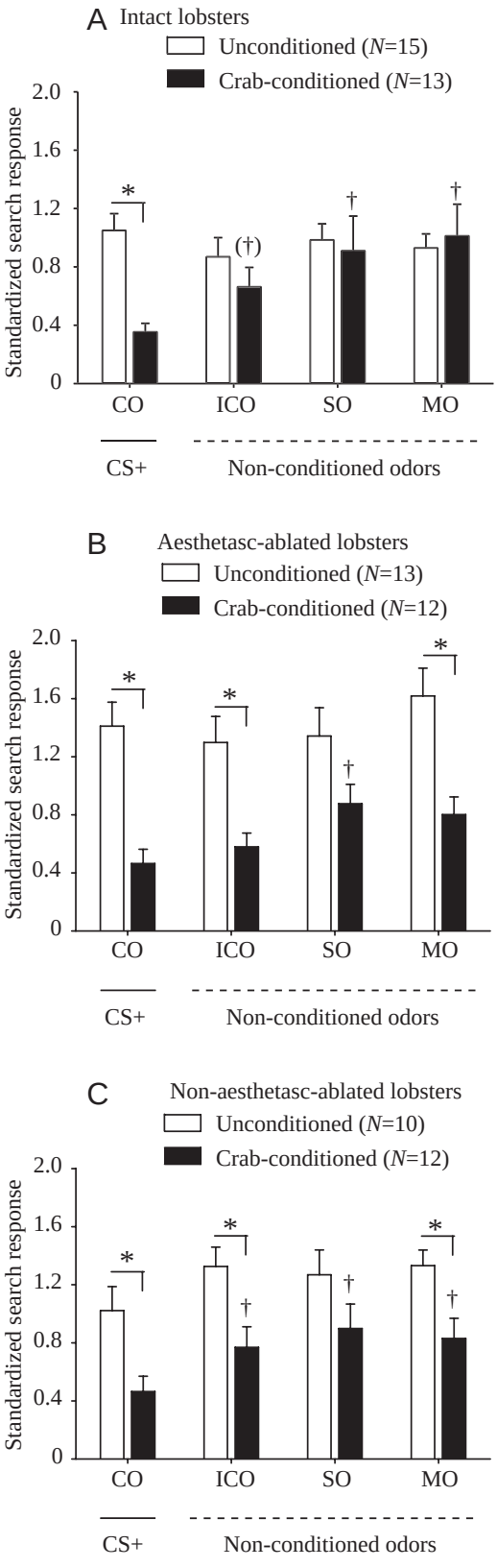

Fig. 4. The ability of intact (sham control) lobsters (A), aesthetasc-ablated lobsters (B) and non-aesthetasc-ablated lobsters (C) to learn an aversive associative task and to discriminate between an aversively conditioned odor (CS+=crab odor, CO) and three other complex odor mixtures (inverse crab odor, ICO; shrimp odor, SO; mullet odor, MO) following aversive conditioning with generalization testing (see Table 2 for protocol). Values are means + S.E.M. *Search responses significantly different in unconditioned and conditioned lobsters (planned-comparisons one-way ANOVA, $P<0.05$). †Search responses significantly larger than those elicited by crab odor in conditioned lobsters (planned-comparisons one-way ANOVA, $P<0.05$). (†) Search responses close to being significantly different from those elicited by crab odor in conditioned lobsters (planned-comparisons one-way ANOVA, $0.05<P<0.10$). For the planned comparisons, critical values for a 5% experiment-wise error rate were determined by the sequential Bonferroni test using the Dunn–Šidák method (Sokal and Rohlf, 1998). For a description of search responses and calculation of standardized search responses relative to the responses to oyster extract in the preconditioning phase, see Materials and methods.

of aesthetascs as independent variables, and odor type as dependent variable; no effect of the presence of aesthetascs: $F_{1,21}$=1.972, P=0.175; no interactions between the presence of aesthetascs and any of the other variables, P>0.05).

Non-aesthetasc-ablated lobsters

The results in Fig. 4C show that non-aesthetasc-ablated lobsters learned to avoid the conditioned odor CO and could discriminate CO from the novel non-conditioned odors but also generalized to some extent between these odors. Unconditioned non-aesthetasc-ablated lobsters did not respond differently to the four complex odor mixtures (one-way ANOVA with odors as repeated measures, $F_{3,36}$=0.994, P=0.407) (Fig. 4C). They searched for 25±1 s (mean ± S.E.M., N=40) after odor stimulation. Non-aesthetasc-ablated lobsters that were conditioned to avoid CO responded less to CO and the non-conditioned odors than did unconditioned non-aesthetasc-ablated lobsters (Fig. 4C). A two-way ANOVA (conditioning treatment as independent variable, odor type as dependent variable) revealed a significant conditioning effect ($F_{1,119}$=7.215, P=0.0146) and a significant odor effect ($F_{3,57}$=6.147, P=0.0011). Responses to CO were significantly smaller in conditioned than in unconditioned non-aesthetasc-ablated lobsters (planned-comparisons one-way ANOVA, P<0.05, Fig. 4C). This was also true for the non-conditioned odors ICO and MO (planned-comparisons one-way ANOVA, P<0.05, Fig. 4C). For conditioned non-aesthetasc-ablated lobsters, responses to CO were significantly smaller than those to the novel non-conditioned odors ISO, SO and MO (planned-comparisons one-way ANOVA, P<0.05, Fig. 4).

Comparisons between intact, aesthetasc-ablated and non-aesthetasc-ablated lobsters

A three-way ANOVA using data from intact, aesthetasc-ablated and non-aesthetasc-ablated lobsters (ablation and conditioning treatments as independent variables, odor type as dependent variable) showed no statistically significant ablation effect ($F_{2,69}$=1.455, P=0.241) and no significant interactions between ablation treatments and any of the other variables (P>0.05). These results indicate that overall responses of intact, aesthetasc-ablated and non-aesthetasc-ablated lobsters were not significantly different from each other. However, as summarized in Fig. 5, both groups of ablated lobsters tended to generalize between the conditioned odors and the novel non-conditioned odors more than did intact lobsters. Furthermore, although all groups of lobsters could discriminate some of the complex odors, aesthetasc-ablated lobsters tended to have the greatest difficulty. This analysis reflects the results of Fig. 4: aesthetasc-ablated lobsters only significantly discriminated SO from the aversively conditioned CO, whereas both intact and non-aesthetasc-ablated lobsters tended to clearly discriminate all novels odors (SO, MO and ICO) from the conditioned odor CO.

Odor learning and discrimination of blend ratios of a binary mixture

The conditioning paradigm described in the previous section

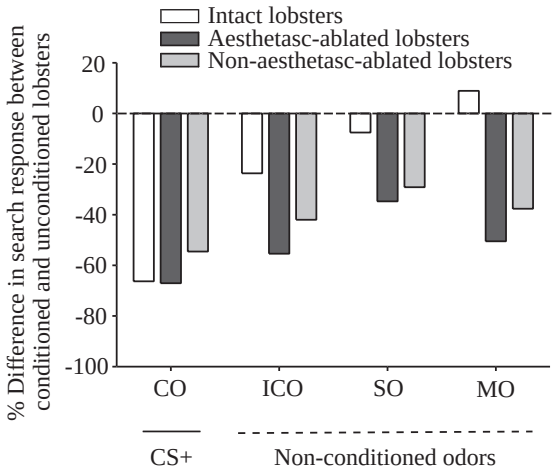

Fig. 5. Percentage change in search responses to the aversively conditioned odor CS+ (crab odor, CO) and the novel non-conditioned odors (inverse crab odor, ICO; shrimp odor, SO; mullet odor, MO) following an aversive conditioning with generalization testing (protocol of Table 2) in intact lobsters, aesthetasc-ablated lobsters and non-aesthetasc-ablated lobsters. For each group of lobsters, the percentage change in search responses to an odorant following conditioning is the percentage difference between responses of conditioned lobsters and unconditioned lobsters relative to responses of unconditioned lobsters. Data are from Fig. 4.

(Table 2) is particularly useful in assessing perceptual similarities between the CS+ and novel non-conditioned odors. Results from that paradigm suggested that all lobsters, but particularly aesthetasc-ablated lobsters, had difficulty discriminating between two complex odor mixtures that differed only in the ratio of their components (CO *versus* ICO) (Fig. 4B). Therefore, we challenged intact lobsters and aesthetasc-ablated lobsters with the task of discriminating between blend ratios of the same binary mixture, AMP+taurine, by using a discrimination conditioning, as described in Table 3. During the conditioning phase of this paradigm, we not only aversively conditioned one blend ratio of AMP+taurine (=CS+), but other blend ratios of the same mixture were also presented specifically unpaired to the US to allow lobsters to associate these odors as safe stimuli (=CS–). Therefore, this paradigm emphasized the perceptual differences between the CS+ and CS– blend ratios, so that the discrimination abilities could be more easily evaluated.

Intact lobsters

The results in Fig. 6A show that intact lobsters learned this aversive odor-associative task and discriminated the CS+ blend ratio 99.9:0.1 from the CS– blend ratios. Discrimination-conditioned intact (sham control) lobsters responded slightly less to the CS+ AMP:taurine 99.9:0.1 blend ratio than did unconditioned intact lobsters (Fig. 6A). In contrast, conditioned intact lobsters responded more to the three CS– blend ratios (99:1, 90:10 and 50:50) than did unconditioned intact lobsters (Fig. 6A). A two-way ANOVA (conditioning

treatment as independent variable, blend ratio as dependent variable) revealed a significant conditioning effect ($F_{1,32}$=5.211, P=0.0292), a significant blend ratio effect ($F_{3,96}$=13.199, P<0.000001) and a significant interaction effect between conditioning treatments and blend ratios ($F_{3,96}$=4.491, P=0.0054). However, only responses to the blend ratio 90:10 were significantly different in unconditioned and conditioned intact lobsters (planned-comparisons one-way ANOVA, P<0.05, Fig. 6A). Furthermore, the responses of conditioned intact lobsters to the CS+ blend ratio were significantly smaller than those to the three CS– blend ratios (planned-comparisons one-way ANOVA, P<0.05, Fig. 6A). Finally, conditioning did not affect responses to oyster extract (OE), since responses to OE before and after conditioning were not significantly different (*t*-test for dependent samples, P>0.05; Fig. 6A).

Aesthetasc-ablated lobsters

The results in Fig. 6B show that aesthetasc-ablated lobsters learned this aversive odor-associative task and discriminated the CS+ blend ratio 99.9:0.1 from the CS– blend ratios. Aesthetasc-ablated lobsters subjected to discrimination conditioning responded less to the CS+ blend ratio than did unconditioned aesthetasc-ablated lobsters (Fig. 6B). In contrast, conditioned aesthetasc-ablated lobsters responded more to the CS– blend ratios than did unconditioned aesthetascs-ablated lobsters (Fig. 6B). A two-way ANOVA (conditioning treatment as independent variable, blend ratio as dependent variable) revealed a significant conditioning effect ($F_{1,27}$=4.215, P=0.0499), a significant blend ratio effect ($F_{3,81}$=4.730, P=0.0432) and a significant interaction effect between blend ratio and conditioning ($F_{3,81}$=7.046, P=0.00029). However, only responses to the blend ratio 50:50 were significantly different in unconditioned and conditioned aesthetasc-ablated lobsters (planned-comparison one-way ANOVA, P<0.05, Fig. 6B). Furthermore, the responses of conditioned aesthetasc-ablated lobsters were significantly smaller to the CS+ than to the CS– blend ratios (planned-comparisons one-way ANOVA, P<0.05, Fig. 6B). Finally, conditioning did not affect responses to oyster extract (OE) because the responses to OE before and after conditioning were not significantly different (*t*-test for dependent samples, P>0.05; Fig. 6B). The ability of aesthetasc-ablated animals to learn this task and to discriminate among these odorants is probably not due to incomplete ablations because aesthetasc-ablated lobsters with no aesthetascs did not behave differently from those with a few remaining aesthetascs (three-way ANOVA with conditioning treatment and the presence of aesthetascs as independent variables, and odor type as dependent variable; no effect of the presence of aesthetascs: $F_{1,25}$=0.036, P=0.850, no interactions between the presence of aesthetascs and any of the other variables, P>0.05).

Comparisons between intact lobsters and aesthetasc-ablated lobsters

Fig. 7 presents a summary of the data of Fig. 6 showing that removal of aesthetascs did not impair the lobster's ability to

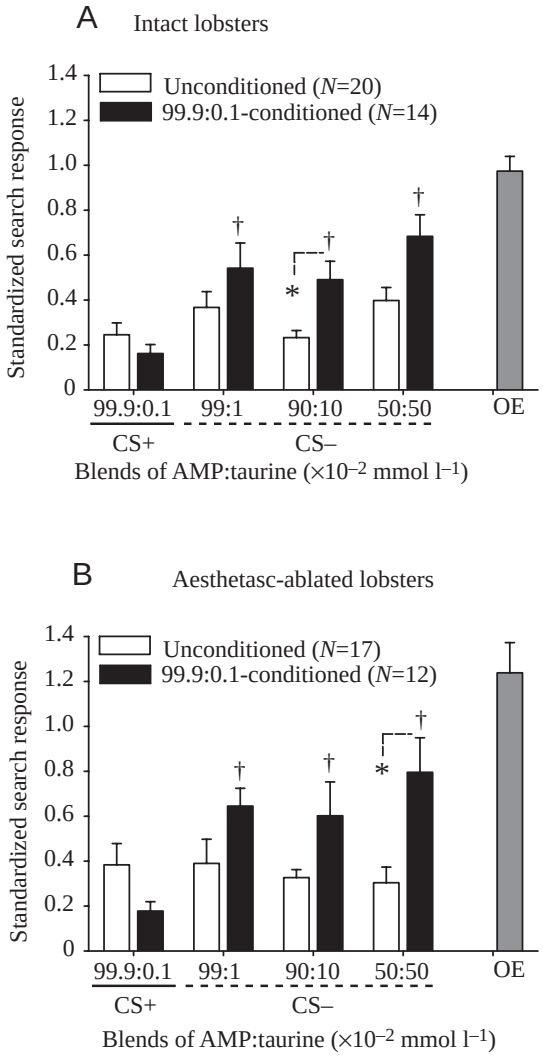

Fig. 6. The ability of intact (sham control) lobsters (A) and aesthetasc-ablated lobsters (B) to learn a discrimination conditioning task and to discriminate between binary mixtures of AMP+taurine with the same total concentration (1 mmol l^{-1}) but at different blend ratios. The discrimination conditioning paradigm is described in Table 3. The aversively conditioned odorant (CS+) was the 99.9:0.1 blend ratio, and the conditioned 'safe' odorants (CS–) were the blend ratios 99:1, 90:10 and 50:50. Values are means + S.E.M. *Search responses significantly different in unconditioned and conditioned lobsters (planned comparisons one-way ANOVA, P<0.05); †Search responses significantly larger than those elicited by the aversively conditioned blend ratio 99.9:0.1 (CS+) in conditioned lobsters (planned-comparisons one-way ANOVA, P<0.05). For the planned comparisons, critical values for a 5% experiment-wise error rate were determined by the sequential Bonferroni test using the Dunn–Šidák method (Sokal and Rohlf, 1998). Search responses of conditioned lobsters to oyster extract (OE) after the post-conditioning phase are also shown. In both intact and aesthetasc-ablated lobsters, search responses to oyster extract before and after conditioning were not significantly different (P<0.05, *t*-test for dependent samples). For a description of search responses and calculation of the standardized search responses relative to the responses to oyster extract in the preconditioning phase, see Materials and methods.

discriminate the 99.9:0.1 blend ratio from 99:1, 90:10 and 50:50 blend ratios. A three-way ANOVA that included data from both intact lobsters and aesthetasc-ablated lobsters (ablation and conditioning treatments as independent variables, blend ratio as dependent variable) showed no statistically significant ablation effect ($F_{1,59}$=1124, P=0.293) and no interactions between ablation treatment and any of the other variables (P>0.30).

Discussion

Our results show that spiny lobsters lacking either aesthetascs or non-aesthetasc setae are capable of learning two different aversive conditioning tasks and discriminating between closely related odorant mixtures. However, their performance in odor discrimination was slightly poorer, particularly that of aesthetasc-ablated lobsters, than that of intact animals. These results demonstrate that the two antennular sensory pathways – the aesthetasc/olfactory lobe (OL) pathway and the non-aesthetasc/lateral antennular neuropil (LAN) pathway – have significant functional redundancy and overlap. These pathways also have some complementary functions. In the following section, we discuss these topics and the basis by which spiny lobsters perceive differences in complex odorant mixtures.

The role of parallel chemosensory pathways

Parallel pathways mediate odor-associative learning

Odor-associative learning can be mediated by either the aesthetasc/OL pathway or the non-aesthetasc/LAN pathway.

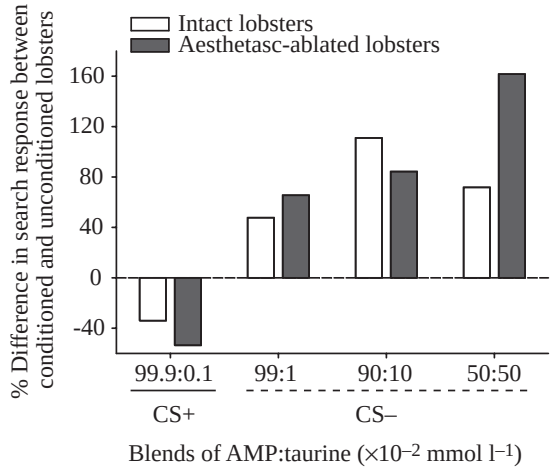

Fig. 7. Percentage change in search responses to the aversively conditioned (CS+) blend ratio of AMP:taurine mixture (99.9:0.1 blend ratio) and the conditioned safe (CS–) blend ratios 99:1, 90:10 and 50:50 following a discrimination conditioning paradigm in intact and aesthetasc-ablated lobsters. For each group of lobsters, the percentage change in search responses to an odorant following conditioning is the percentage difference between responses of conditioned lobsters and unconditioned lobsters relative to responses of unconditioned lobsters. Data are from Fig. 6.

The aesthetasc pathway is sufficient for odor-associative learning, as is the non-aesthetasc pathway, and neither pathway is alone necessary. All groups of lobsters – intact, aesthetasc-ablated and non-aesthetasc-ablated – learned to avoid an aversively conditioned odorant, and they learned the task equally well. This was true for learning to avoid a 32-component mixture (crab odor; Figs 4, 5) or a binary mixture (AMP+taurine; Figs 6, 7).

A critical role for the aesthetasc/OL pathway in olfactory learning was expected since it is a prominent chemosensory pathway that has features of other olfactory pathways showing plasticity. Thus, the OLs have a glomerular organization reminiscent of the olfactory pathways of vertebrates, insects and gastropods (Tolbert and Hildebrand, 1981; Mellon and Munger, 1990; Sandeman et al., 1992; Chase and Tolloczko, 1993; Hildebrand and Shepherd, 1997; Schmidt and Ache, 1996b; Mori et al., 1999; Laissue et al., 1999), species with powerful olfactory learning (Croll and Chase, 1980; Gelperin, 1990; Eichenbaum and Otto, 1993; Slotnick, 1994; Smith, 1996; Faber et al., 1999; Derby, 2000; Derby et al., 2001). The neuronal structures along the 'OL pathway' that are implicated in learning are not known but may include the terminal medulla (including the hemiellipsoid bodies) and/or the accessory lobes, which are complex neuropils that receive extensive inputs from the OL. These neuropils are composed of glomeruli and are involved in higher-order multimodal sensory integration (Maynard, 1966; Blaustein et al., 1988; Derby and Blaustein, 1988; Schmidt and Ache, 1996a,b; Mellon and Alones, 1997; Mellon, 2000). Unfortunately, studies of the behavioral roles of these neuropils are few. The terminal medulla has been implicated in modulating feeding behavior in response to chemical and tactile stimuli (Maynard and Sallee, 1970; Hazlett, 1971; Sears et al., 1991) and in pheromone-mediated courtship in crabs (Gleeson et al., 1987). The terminal medulla and hemiellipsoid body have been suggested to be involved in associative learning and memory, mostly because their organization and connectivity are similar to those of the mushroom bodies of insects (Maynard, 1966; Maynard and Sallee, 1970; Blaustein et al., 1988), which are involved in odor learning (Davis, 1993; Strausfeld et al., 1998). Finally, the accessory lobes may be involved in odor processing in spatially complex environments (Wachowiak et al., 1996), but there are no direct tests of this idea.

Our results showing that the non-aesthetasc/LAN pathway is sufficient for olfactory learning is more surprising, since this pathway has usually been considered to function in sensory-motor reflexes and not in more complex behaviors (Maynard, 1966; Schmidt and Ache, 1993, 1996a). Odor-associative learning *via* the LANs may also implicate the terminal medulla, which receives input from LAN output interneurons (Derby and Blaustein, 1988; Mellon and Alones, 1994; Schmidt and Ache, 1996a).

Parallel pathways and olfactory discrimination

Olfactory discrimination can be mediated by either the aesthetasc/OL pathway or the non-aesthetasc/LAN pathway.

We demonstrate this for two sets of odor mixtures with highly similar compositions: for complex mixtures that mimic natural foods (Figs 4, 5) and for blend ratios of a binary mixture (Figs 6, 7).

The finding that the aesthetasc/OL pathway is sufficient to mediate olfactory discrimination was expected for several reasons. First, aesthetasc chemosensory neurons, which are the only identified chemosensory inputs to the glomerularly organized OLs (Mellon and Munger, 1990; Schmidt and Ache, 1992, 1996b; Sandeman and Sandeman, 1994), have a diversity of response spectra for many biologically important odors (Fadool et al., 1993; Michel and Ache, 1994; Simon and Derby, 1995). Second, each aesthetasc is innervated by neurons with different response spectra (Steullet et al., 2000), and each glomerulus receives input from neurons from different aesthetascs (Mellon and Munger, 1990). This suggests that odorant quality is represented in the OL as an across-glomerular spatial map (Mellon and Munger, 1990; Wachowiak and Ache, 1998), similar to the vertebrate olfactory bulb and the insect antennal lobe (Cinelli et al., 1995; Friedrich and Korsching, 1998; Galizia et al., 1999; Sachse et al., 1999). Thus, the glomerular organization of the crustacean OL might enable odor recognition and discrimination.

Our finding that the non-aesthetasc/LAN pathway is sufficient for olfactory discrimination demonstrates an alternative to the aesthetasc/OL pathway. Some neural components of this pathway have already been described. The odor responsiveness of non-aesthetasc antennular chemosensory neurons has been partially characterized in electrophysiological studies. These neurons are activated by many of the same food-related odors that stimulate aesthetasc neurons, and they have a diversity of response spectra (Fuzessery, 1978; Tierney et al., 1988; Cate and Derby, 2002a). In addition, interneurons ascending from the LANs to the terminal medulla have a variety of response specificities and appear to be sufficient to form an odor-specific neural code (Derby and Blaustein, 1988; Schmidt and Ache, 1996b). This overlap in tuning of peripheral and central neurons in the aesthetasc/OL pathway and the non-aesthetasc/LAN pathway may explain the similarity in responsiveness of aesthetasc-ablated lobsters and non-aesthetasc-ablated lobsters in our studies.

Although we cannot completely rule out the possibility that some non-aesthetasc chemosensory neurons project into the OLs and that these are sufficient to allow aesthetasc-ablated lobsters to discriminate odors *via* the OL pathway, we believe that non-aesthetasc chemosensory input to the LANs provides lobsters with sufficient information to discriminate odors. Interneurons with dense arborizations in the OLs and small branches in the LANs (Schmidt and Ache, 1996b; Mellon and Alones, 1994) may furthermore provide coupling and cross-talk between the two pathways that might be important in odor discrimination, odor generalization and other odor processing.

Our results on olfactory discrimination from the generalization assay (Table 2; Figs 4, 5) suggest, however, that the aesthetasc/OL pathway and non-aesthetasc/LAN pathway are not completely equivalent and that they complement each other to produce behavior not possible with only one pathway. In the aversive conditioning with generalization testing paradigm, ablated lobsters tended to show more generalization between a complex odor and novel but related complex odors than did intact lobsters (Figs 4, 5). This suggests that both aesthetasc and non-aesthetasc chemosensory neurons are complementary and necessary to enhance discrimination and reduce generalization. Furthermore, intact animals and non-aesthetasc-ablated animals tended to discriminate slightly better between closely related complex mixtures than did aesthetasc-ablated animals. Although further experiments are needed to confirm this trend, these data suggest that processing of aesthetasc chemosensory inputs through the glomerular OLs and/or accessory lobes provides additional contrast enhancement for closely related complex odorant mixtures. Similarly, in the honeybee *Apis mellifera*, the local circuitry of the glomerularly organized antennal lobes facilitates discrimination of related odorants since functional disruption of this circuitry impairs discrimination of structurally related odorant compounds but not highly divergent odorant compounds (Stopfer et al., 1997).

Our results from the discrimination conditioning assay (Table 3; Figs 6, 7) show that both intact animals and aesthetasc-ablated animals effectively discriminate among highly related odorants – different blend ratios of the binary mixture AMP+taurine. In contrast, intact lobsters generalized completely between different blend ratios of the mixture AMP+taurine following the aversive conditioning with generalization protocol of Table 2 (data not shown). This suggests that discrimination conditioning implements mechanisms of neural plasticity that allow further contrast sharpening between odor stimuli, and that this may even occur along the non-aesthetasc/LAN pathway. In honeybees, odor-associative learning transforms the neural activity pattern generated by a rewarded odor, making it less similar to an unrewarded odor; in this case, the transformations occurred in the glomerularly organized antennal lobes (Faber et al., 1999).

Why have two antennular chemosensory pathways with partially overlapping function?

The present study provides the first evidence for extensive redundancy and overlap in the function of aesthetasc and non-aesthetasc chemosensory neurons in odor-associative learning and odor discrimination. Functional overlap between these populations of antennular chemosensory neurons also occurs for odor-mediated activation and orientation (Horner et al., 2000; Derby et al., 2001; Steullet et al., 2001). The overlap in function of these two antennular chemosensory pathways can be advantageous to animals. The two pathways may be redundant partial back-up systems in case the antennules, and particularly the aesthetasc regions, are damaged (Harrison et al., 2001). The output from the two pathways might be integrated at some higher neural level, thus increasing sensitivity or accuracy (Van Drongelen et al., 1978; Meisami,

1989) and reducing odor generalization (this paper) beyond that possible by either pathway alone.

The two pathways are known to have some distinct functions. For instance, glutamate-mediated antennular grooming behavior in lobsters (Barbato and Daniel, 1997; Daniel et al., 2000) and pheromone-mediated courtship display behavior in male blue crabs *Callinectes sapidus* (Gleeson, 1982) are driven exclusively by chemosensory neurons from aesthetascs and/or asymmetric setae. Thus, this suggests that pheromones in spiny lobsters (Zimmer-Faust et al., 1985; Ratchford and Eggleston, 1998, 2000) might be detected by aesthetasc but not by non-aesthetasc chemosensory neurons. However, other functional differences between these two pathways remain to be examined. For example, the bimodal chemotactile non-aesthetasc setae and the LAN pathway may be unique in providing information about the location of stimulation on the antennule or on other parts of the body (second antennae and carapace) since some output neurons from the LAN arborize in the median antennular neuropil, antennal neuropil and tegumentary neuropil (Schmidt and Ache, 1996a). Finally, the two pathways might detect different qualitative, quantitative and temporal features of the chemical signals; these differences might be revealed *via* different and even more complex behavioral experiments than those described in the present work.

How do lobsters perceive differences in the compositions of odorant mixtures?

Our results show that lobsters can discriminate among mixtures with highly related compositions. Thus, lobsters can discriminate among multi-component mixtures that differ both in blend ratio and in number of components (CO *versus* SO and MO), among multi-component mixtures that differ only in blend ratios and not in components (CO *versus* ICO) and among binary mixtures that differ only in blend ratios of a single binary mixture (AMP+taurine at 99.9:0.1 *versus* 99:1, 90:10 and 50:50). These results are consistent with an earlier study using discrimination conditioning (Fine-Levy et al., 1989).

Furthermore, lobsters also generalize between mixtures, and the extent of generalization depends on the similarity in the composition of the mixtures. Thus, lobsters generalize more between complex mixtures that share all the same components but differ markedly in their blend ratios (CO *versus* ICO) than they do between mixtures that have unique components but whose common components have relatively similar blend ratios (CO *versus* MO or SO). To understand this point, similarities in the compositions of CO, ICO, MO and SO must be qualitatively and quantitatively compared. ICO contains the same 32 components as CO, with the following difference: the component in ICO with the highest concentration is the component in CO with the lowest concentration; the component in ICO with the second highest concentration is the component in CO with the second lowest concentration; and so forth, until the component in ICO with the lowest concentration is the component in CO with the highest concentration. Thus, CO and ICO contain exactly the same compounds but at different blend ratios. In contrast, SO differs from CO in two ways: SO lacks three chemical compounds present in CO, and the 29 components common to both have relatively small differences in blend ratio. Eleven compounds were not common to MO and CO, and there were large differences in the blend ratios of some of their 28 common components. The relative degree of similarity between the chemical composition of CO, ICO, SO and MO can be evaluated more quantitatively with hierarchical cluster analysis (joining-tree clustering; Statistica, StatSoft Inc, Tulsa, OK, USA). Cluster analysis joins together objects (in this case, mixtures) into successively larger groups using a measure of dissimilarity (in this case, the difference in concentration of each component in the mixtures). Using a measure of dissimilarity that emphasizes the relative differences in the blend ratios (i.e. 1 minus the Pearson *r* correlation), the cluster analysis clearly indicated that ICO was most different from the other mixtures (Fig. 8B). In contrast, using a measure of dissimilarity that emphasizes the presence or absence of an odorant component (i.e. percentage disagreement method), the analysis showed that MO was most different from the other mixtures (Fig. 8A).

The pattern of generalization shown by the lobsters is more similar to that expected if lobsters were evaluating the presence

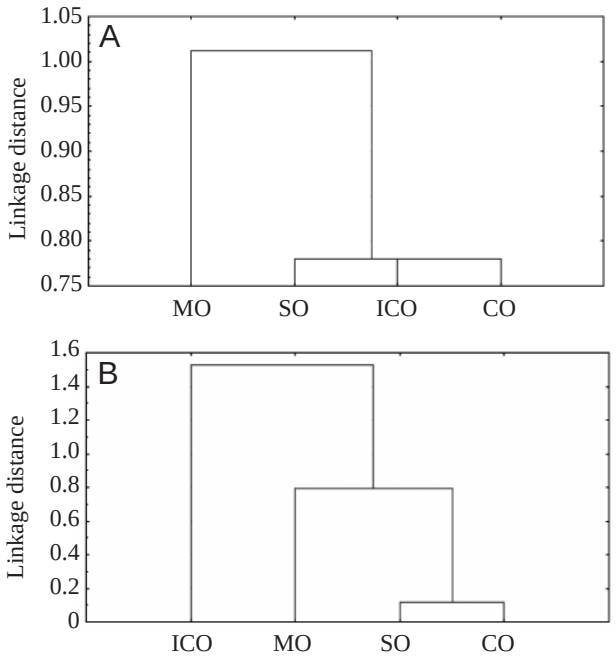

Fig. 8. Results of two cluster analyses showing the relative similarity of the chemical composition of the complex odor mixtures crab odor (CO), inverse crab odor (ICO), shrimp odor (SO) and mullet odor (MO). (A) Cluster analysis that uses 'percentage of disagreement' as the dissimilarity measure, which emphasizes the components unique to the odors (i.e. the presence or absence of components). (B) Cluster analysis that uses '1 minus the Pearson *r* correlation' as the dissimilarity measure, which emphasizes differences in the relative concentrations of each component.

or absence of components in a mixture (analogous to the percentage disagreement method for measuring dissimilarity of mixtures in the cluster analysis of Fig. 8A) rather than relative differences in the blend ratios (analogous to the correlation method for measuring dissimilarity of mixtures in the cluster analysis of Fig. 8B). Such generalization between odors that differ only in the ratios of the components may reflect a survival adaptation rather than limited processing capabilities. Since the quality and intensity of any odor stimulus with a defined contextual meaning (e.g. a crab odor for a hungry lobster) vary over time and space, an animal would benefit from being able to filter out small and irrelevant differences in composition. However, the extent of generalization between related mixtures is variable because of the salience of the mixtures. Salience can come in many forms; one type is represented in our discrimination conditioning protocol (Table 3), in which the salience of the difference between two mixtures (designated as CS+ and CS–) is emphasized. In this case, lobsters showed very little generalization between different blend ratios of AMP+taurine (Figs 6, 7). Yet, with only aversive conditioning (protocol in Table 2, with a CS+ but no CS–), there was extensive generalization between these same blend ratios (data not shown). This suggests that small differences in mixtures are detected by lobsters but that those differences are only acted on when made behaviorally relevant. This idea is supported by physiological studies showing that different blend ratios of AMP+taurine evoke distinct across-neuron patterns (ANPs) from populations of antennular chemosensory neurons and, furthermore, that the ANPs for these blend ratios are more similar to each other than they are to ANPs for other binary mixtures (AMP+glutamate or glutamate+taurine) (Steullet and Derby, 1997).

In conclusion, our results show that learning odor-associative tasks and odor discrimination can be mediated either by the aesthetasc/olfactory lobe pathway or the non-aesthetasc/lateral antennular neuropil pathway of the antennules. In addition, some complementary functions exist between the two pathways. A complete understanding of olfactory processing in lobsters will require further behavioral, anatomical and physiological studies of both chemosensory pathways, including characterizing the central projections of the various non-aesthetasc antennular chemosensory neurons, the mechanisms of central sensory processing in both pathways, the nature of cross-talk between the pathways and the involvement of chemosensory neurons on the second antennae, carapace and legs in odor discrimination and other odor processing.

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References

Barbato, J. C. and Daniel, P. C. (1997). Chemosensory activation of an antennular grooming behavior in the spiny lobster, *Panulirus argus*, is tuned narrowly to L-glutamate. *Biol. Bull.* **293**, 107–115.

Belluscio, L. and Katz, L. C. (2001). Symmetry, stereotypy and topography of odorant representations in mouse olfactory bulbs. *J. Neurosci.* **21**, 2113–2122.

Blaustein, D. N., Derby, C. D., Simmons, R. B. and Beall, A. C. (1988). Structure of the brain and medulla terminalis of the spiny lobster *Panulirus argus* and the crayfish *Procambarus clarkii*, with an emphasis on olfactory centers. *J. Crust. Biol.* **8**, 493–519.

Blaustein, D. N., Simmons, R. B., Burgess, M. F., Derby, C. D., Nishikawa, M. and Olson, K. S. (1993). Ultrastructural localization of 5′AMP receptor sites on the dendrites of olfactory receptor cells of the spiny lobster. *J. Neurosci.* **13**, 2821–2828.

Boeckh, J., Ernst, K.-D., Sass, H. and Waldow, U. (1984). Anatomical and physiological characteristics of individual neurons in the central antennal pathways of insects. *J. Insect Physiol.* **30**, 15–26.

Carr, W. E. S. and Derby, C. D. (1986). Chemically stimulated feeding behavior in marine animals: importance of chemical mixtures and involvement of mixture interactions. *J. Chem. Ecol.* **12**, 989–1011.

Cate, H. S. and Derby, C. D. (2000). A novel chemo-/mechanosensillum that is widely distributed on the Caribbean spiny lobster and other lobsters. *Chem. Senses* **25**, 633–634 (Abstract).

Cate, H. S. and Derby, C. D. (2001). Morphology and distribution of setae on the antennules of the Caribbean spiny lobster *Panulirus argus* reveal new types of bimodal chemo-mechanosensilla. *Cell Tissue Res.* **304**, 439–454.

Cate, H. S. and Derby, C. D. (2002a). Ultrastructure and physiology of the hooded sensillum, a bimodal chemo-mechanosensillum of lobsters. *J. Comp. Neurol.* **442**, 293–307.

Cate, H. S. and Derby, C. D. (2002b). Hooded sensilla homologues: structural variation of a widely distributed biomodal chemo-mechanosensillum. *J. Comp. Neurol.* **444** (in press).

Chase, R. and Tolloczko, B. (1993). Tracing neural pathways in snail olfaction: from the tip of the tentacles to the brain and beyond. *Microsc. Res. Tech.* **24**, 214–230.

Cinelli, A. R., Hamilton, K. A. and Kauer, J. S. (1995). Salamander olfactory-bulb neuronal-activity observed by video-rate, voltage-sensitive dye imaging. III. Spatial and temporal properties of responses evoked by odorant stimulation. *J. Neurophysiol.* **73**, 2053–2071.

Croll, R. P. and Chase, R. (1980). Plasticity of olfactory orientation to foods in the snail *Achatina fulica*. *J. Comp. Physiol. A* **136**, 267–277.

Cromarty, S. I. and Derby, C. D. (1997). Multiple excitatory receptor types on individual olfactory neurons: implications for coding of mixtures in the spiny lobster. *J. Comp. Physiol. A* **180**, 481–491.

Daniel, P. C., Shineman, M. and Fischetti, M. (2002). Comparison of chemosensory activation of antennular grooming behavior in five species of decapods. *Mar. Freshwater Res.* **52**, 1333–1338.

Davis, R. L. (1993). Mushroom bodies and *Drosophila* learning. *Neuron* **11**, 1–14.

Derby, C. D. (1995). Single unit electrophysiological recording techniques from crustacean chemoreceptor neurons. In *CRC Handbook on Experimental Cell Biology of Taste and Olfaction: Current Techniques and Protocols* (ed. A. I. Spielman and J. D. Brand), pp. 241–250. Boca Raton: CRC Press.

Derby, C. D. (2000). Learning from spiny lobsters about chemosensory coding of mixtures. *Physiol. Behav.* **69**, 203–209.

Derby, C. D. and Atema, J. (1982). The function of chemo- and mechanoreceptors in lobster (*Homarus americanus*) feeding behaviour. *J. Exp. Biol.* **98**, 317–327.

Derby, C. D. and Blaustein, D. N. (1988). Morphological and physiological characterization of individual olfactory interneurons connecting the brain and eyestalk ganglia of the crayfish. *J. Comp. Physiol. A* **163**, 777–794.

Derby, C. D., Girardot, M.-N. and Daniel, P. C. (1991). Responses of olfactory receptor cells of spiny lobsters to binary mixtures. I. Intensity mixture interactions. *J. Neurophysiol.* **66**, 112–130.

Derby, C. D., Horner, A. J., Cate, H. S. and Steullet, P. (2001). The sensory basis to feeding behavior in the Caribbean spiny lobster *Panulirus argus*. *Mar. Freshwater Res.* **52**, 1339–1350.

Devine, D. V. and Atema, J. (1982). Function of chemoreceptor organs in spatial orientation of the lobster, *Homarus americanus*: differences and overlap. *Biol. Bull.* **163**, 144–153.

Eichenbaum, H. and Otto, T. (1993). Odor-guided learning and memory in rats: is it 'special'? *Trends Neurosci.* **16**, 22–24.

Faber, T., Joerges, J. and Menzel, R. (1999). Associative learning modifies neural representations of odors in the insect brain. *Nature Neurosci.* **2**, 74–78.

Fadool, D. A., Michel, W. C. and Ache, B. W. (1993). Odor sensitivity of cultured lobster olfactory receptor neurons is not dependent on process formation. *J. Exp. Biol.* **174**, 215–233.

Fine-Levy, J. B., Daniel, P. C., Girardot, M.-N. and Derby, C. D. (1989). Behavioral resolution of quality of odorant mixtures by spiny lobsters: differential aversive conditioning of olfactory responses. *Chem. Senses* **14**, 503–524.

Friedrich, R. W. and Korsching, S. I. (1998). Chemotopic, combinatorial and noncombinatorial odorant representations in the olfactory bulb revealed using a voltage-sensitive axon tracer. *J. Neurosci.* **18**, 9977–9988.

Fuzessery, Z. M. (1978). Quantitative stimulation of antennular chemoreceptors of the spiny lobster, *Panulirus argus*. *Comp. Biochem. Physiol.* **60**, 303–308.

Galizia, C. G., Menzel, R. and Hölldobler, B. (1999). Optical imaging of odor-evoked glomerular activity patterns in the antennal lobes of the ant *Camponotus rufipes*. *Naturwissenschaften* **86**, 533–537.

Gelperin, A. (1990). A taste for learning. *Am. Zool.* **30**, 549–558.

Giri, T. and Dunham, D. W. (1999). Use of the inner antennule ramus in the localization of distant food odours by *Procambarus clarkii* (Girard, 1852) (Decapoda, Cambaridae). *Crustaceana* **72**, 123–127.

Giri, T. and Dunham, D. W. (2000). Female crayfish (*Procambarus clarkii* (Girard, 1852)) use both antennular rami in the localization of male odour. *Crustaceana* **73**, 447–458.

Gleeson, R. A. (1982). Morphological and behavioral identification of the sensory structures mediating pheromone reception in the blue crab, *Callinectes sapidus*. *Biol. Bull.* **163**, 162–171.

Gleeson, R. A., Adams, M. A. and Smith III, A. B. (1987). Hormonal modulation of pheromone-mediated behavior in a crustacean. *Biol. Bull.* **172**, 1–9.

Gleeson, R. A., Hammar, K. and Smith, P. J. S. (2000). Sustaining olfaction at low salinities: mapping ion flux associated with the olfactory sensilla of the blue crab *Callinectes sapidus*. *J. Exp. Biol.* **203**, 3145–3152.

Gleeson, R. A., McDowell, L. M. and Aldrich, H. C. (1996). Structure of the aesthetasc (olfactory) sensilla of the blue crab, *Callinectes sapidus*: transformations as a function of salinity. *Cell Tissue Res.* **284**, 279–288.

Grünert, U. and Ache, B. W. (1988). Ultrastructure of the aesthetasc (olfactory) sensilla of the spiny lobster *Panulirus argus*. *Cell Tissue Res.* **251**, 95–103.

Harrison, P. J. H., Cate, H. S., Steullet, P. and Derby, C. D. (2001). Structural plasticity in the olfactory system of adult spiny lobsters: postembryonic development enables life-long growth, turnover and regeneration. *Mar. Freshwater Res.* **52**, 1357–1366.

Hazlett, B. A. (1971). Chemical and chemotactic stimulation of feeding behavior in the hermit crab *Petrochirus diogenes*. *Comp. Biochem. Physiol.* **39A**, 665–670.

Helluy, S. M., Benton, J. L., Langworthy, K. A., Ruchhoeft, M. L. and Beltz, B. S. (1996). Glomerular organization in developing olfactory and accessory lobes of American lobsters: stabilization of numbers and increase in size after metamorphosis. *J. Neurobiol.* **29**, 459–472.

Hildebrand, J. G. and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* **20**, 595–631.

Horner, A. J., Ngo, V., Steullet, P., Keller, T., Weissburg, M. J. and Derby, C. D. (2000). The role of different types of antennular sensilla in orientation by Caribbean spiny lobsters to a natural odor stimulus under controlled flow conditions. *Chem. Senses* **25**, 670 (Abstract).

Laissue, P. P., Reiter, C., Hiesinger, P. R., Halter, S., Fischbach, K. F. and Stocker, R. F. (1999). Three-dimensional reconstruction of the antennal lobe in *Drosophila melanogaster*. *J. Comp. Neurol.* **405**, 543–552.

Livermore, A., Hutson, M., Ngo, V., Hadjisimos, R. and Derby, C. D. (1997). Elemental and configural learning and the perception of odorant mixtures by the spiny lobster *Panulirus argus*. *Physiol. Behav.* **62**, 169–174.

Lyle, W. G. and MacDonald, G. D. (1983). Molt stage determination in the Hawaiian spiny lobster *Panulirus marginatus*. *J. Crust. Biol.* **3**, 208–216.

Lynn, W. H., Meyer, E. A., Peppiatt, C. E. and Derby, C. D. (1994). Perception of odor mixtures by the spiny lobster *Panulirus argus*. *Chem. Senses* **19**, 331–347.

Mackintosh, N. J. (1973). *Conditioning and Associative Learning*. New York: Oxford University Press.

Maynard, D. M. (1966). Integration in crustacean ganglia. *Symp. Soc. Exp. Biol.* **20**, 111–149.

Maynard, D. M. and Sallee, A. (1970). Disturbance of feeding behavior in the spiny lobster, *Panulirus argus*, following bilateral ablation of the medulla terminalis. *Z. Vergl. Physiol.* **66**, 123–140.

McLeese, D. W. (1973). Olfactory responses of lobsters (*Homarus americanus*) to solutions from prey species and to seawater extracts and chemical fractions of fish muscle and effects of antennule ablation. *Mar. Behav. Physiol.* **2**, 237–249.

Meisami, E. (1989). A proposed relationship between increases in the number of olfactory receptor neurons, convergence ratio and sensitivity in the developing rat. *Dev. Brain Res.* **46**, 9–19.

Mellon, D., Jr (2000). Convergence of multimodal sensory input onto higher-level neurons of the crayfish olfactory pathway. *J. Neurophysiol.* **84**, 3043–3055.

Mellon, D., Jr and Alones, V. (1994). Identification of three classes of multiglomerular, broad-spectrum neurons in the crayfish olfactory midbrain by correlated patterns of electrical activity and dendritic arborization. *J. Comp. Physiol. A* **177**, 55–71.

Mellon, D., Jr and Alones, V. (1997). Response properties of higher level neurons in the central olfactory pathway of the crayfish. *J. Comp. Physiol. A* **181**, 205–216.

Mellon, D., Jr and Munger, S. D. (1990). Nontopographic projection of olfactory sensory neurons in the crayfish brain. *J. Comp. Neurol.* **296**, 253–262.

Michel, W. C. and Ache, B. W. (1994). Odor-evoked inhibition in primary olfactory receptor neurons. *Chem. Senses* **19**, 11–24.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J. and Axel, R. (1996). Visualizing an olfactory sensory map. *Cell* **87**, 675–686.

Mori, K., Nagao, H. and Yoshihara, Y. (1999). The olfactory bulb: coding and processing of odor molecule information. *Science* **286**, 711–715.

Mori, K. and Yoshihara, Y. (1995). Molecular recognition and olfactory processing in the mammalian olfactory system. *Prog. Neurobiol.* **45**, 585–620.

Pinching, A. J. and Powell, T. P. S. (1971). The neuropil of the glomeruli of the olfactory bulb. *J. Cell Sci.* **9**, 347–377.

Ratchford, S. G. and Eggleston, D. B. (1998). Size- and scale-dependent chemical attraction contribute to an ontogenetic shift in sociality. *Anim. Behav.* **56**, 1027–1034.

Ratchford, S. G. and Eggleston, D. B. (2000). Temporal shift in the presence of a chemical cue contributes to a diel shift in sociality. *Anim. Behav.* **59**, 793–799.

Reeder, P. B. and Ache, B. W. (1980). Chemotaxis in the Florida spiny lobster, *Panulirus argus*. *Anim. Behav.* **28**, 831–839.

Rescorla, R. A. (1969). Pavlovian conditioned inhibition. *Psychol. Bull.* **72**, 77–94.

Sachse, S., Rappert, A. and Galizia, C. G. (1999). The spatial representation of chemical structures in the antennal lobe of honeybees: steps towards the olfactory code. *Eur. J. Neurosci.* **11**, 3970–3982.

Sandeman, D. C. and Denburg, J. L. (1976). The central projections of chemoreceptor axons in the crayfish revealed by axoplasmic transport. *Brain Res.* **115**, 492–496.

Sandeman, D. C. and Sandeman, R. E. (1994). Electrical responses and synaptic connections of giant serotonin-immunoreactive neurons in crayfish olfactory and accessory lobes. *J. Comp. Neurol.* **341**, 130–144.

Sandeman, D., Sandeman, R., Derby, C. D. and Schmidt, M. (1992). Morphology of the brain of crayfish, crabs and spiny lobsters: a common nomenclature for homologous structures. *Biol. Bull.* **183**, 304–326.

Schmidt, M. and Ache, B. W. (1992). Antennular projections to the midbrain of the spiny lobster. II. Sensory innervation of the olfactory lobe. *J. Comp. Neurol.* **318**, 291–303.

Schmidt, M. and Ache, B. W. (1993). Antennular projections to the midbrain of the spiny lobster. III. Central arborizations of motoneurons. *J. Comp. Neurol.* **336**, 583–594.

Schmidt, M. and Ache, B. W. (1996a). Processing of antennular input in the brain of the spiny lobster, *Panulirus argus*. I. Non-olfactory chemosensory and mechanosensory pathway of the lateral and median antennular neuropils. *J. Comp. Physiol. A* **178**, 579–604.

Schmidt, M. and Ache, B. W. (1996b). Processing of antennular input in the

brain of the spiny lobster, *Panulirus argus*. II. The olfactory pathway. *J. Comp. Physiol. A* **178**, 605–628.

Schmidt, M., Van Ekeris, L. and Ache, B. W. (1992). Antennular projections to the midbrain of the spiny lobster. I. Sensory innervation of the lateral and median antennular neuropils. *J. Comp. Neurol*. **318**, 277–290.

Sears, M. A., Rittschof, D. and O'Brien, T. (1991). Eyestalk factor modulation of chemically stimulated feeding in sand fiddler crabs *Uca pugilator* (Bosc 1802). *J. Exp. Mar. Biol. Ecol.* **152**, 1–13.

Simon, T. W. and Derby, C. D. (1995). Mixture suppression without inhibition for binary mixtures from whole cell patch clamp studies of *in situ* olfactory receptor neurons of the spiny lobster. *Brain Res*. **678**, 213–224.

Slotnick, B. M. (1994). The enigma of olfactory learning revisited. *Neuroscience* **58**, 1–12.

Smith, B. H. (1996). The role of attention in learning about odorants. *Biol. Bull*. **191**, 76–83.

Sokal, R. R. and Rohlf, F. J. (1998). *Biometry. The Principles and Practice of Statistics in Biological Research*. Third edition. New York: W. H. Freeman.

Spencer, M. and Linberg, K. A. (1986). Ultrastructure of aesthetasc innervation and external morphology of the lateral antennule setae of the spiny lobster *Panulirus interruptus* (Randall). *Cell Tissue Res*. **245**, 69–80.

Steullet, P., Cate, H. S., Michel, W. C. and Derby, C. D. (2000). Functional units of a compound nose: aesthetasc sensilla house similar populations of olfactory receptor neurons on the crustacean antennule. *J. Comp. Neurol*. **418**, 270–280.

Steullet, P. and Derby, C. D. (1997). Coding of blend ratios of binary mixtures by olfactory neurons in the Florida spiny lobster, *Panulirus argus*. *J. Comp. Physiol. A* **180**, 123–135.

Steullet, P., Dudar, O., Flavus, T., Zhou, M. and Derby, C. D. (2001). Selective ablation of antennular sensilla on the Caribbean spiny lobster *Panulirus argus* suggests that dual antennular chemosensory pathways mediate odorant activation of search behavior. *J. Exp. Biol.* **204**, 4259–4269.

Stopfer, M., Bhagavan, S., Smith, B. H. and Laurent, G. (1997). Impaired odor discrimination on desynchronization of odor-encoding neural assemblies. *Nature* **390**, 70–74.

Strausfeld, N. J. (1998). Crustacean–insect relationships: the use of brain characters to derive phylogeny amongst segmented invertebrates. *Brain Behav. Evol*. **52**, 186–206.

Strausfeld, N. J., Hansen, Y., Li, R. S. and Ito, K. (1998). Evolution, discovery and interpretations of arthropod mushroom bodies. *Learning Memory* **5**, 11–37.

Tierney, A. J., Voigt, R. and Atema, J. (1988). Response properties of chemoreceptors from the medial antennular filament of the lobster *Homarus americanus*. *Biol. Bull.* **174**, 364–372.

Tolbert, L. P. and Hildebrand, J. G. (1981). Organization and synaptic ultrastructure of glomeruli in the antennal lobes of the moth *Manduca sexta*: a study using thin sections and freeze-fracture. *Proc. R. Soc. Lond. B* **213**, 279–301.

Van Drongelen, W., Holley, A. and Døving, K. B. (1978). Convergence in the olfactory system: quantitative aspects of odour sensitivity. *J. Theor. Biol.* **71**, 39–48.

Wachowiak, M. and Ache, B. W. (1998). Multiple inhibitory pathways shape odor-evoked responses in lobster olfactory projection neurons. *J. Comp. Physiol. A* **182**, 425–434.

Wachowiak, M., Diebel, C. E. and Ache, B. W. (1996). Functional organization of olfactory processing in the accessory lobe of the spiny lobster. *J. Comp. Physiol. A* **178**, 211–226.

Wachowiak, M., Zochowski, M., Cohen, L. B. and Falk, C. X. (2000). The spatial representation of odors by olfactory receptor neuron input to the olfactory bulb is concentration invariant. *Biol. Bull.* **199**, 162–163.

Zimmer-Faust, R. K., Tyre, J. E. and Case, J. F. (1985). Chemical attraction causing aggregation in the spiny lobster, *Panulirus interruptus* (Randall) and its probable ecological significance. *Biol. Bull.* **169**, 106–118.