

Functional Units of a Compound Nose: Aesthetasc Sensilla House Similar Populations of Olfactory Receptor Neurons on the Crustacean Antennule

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ABSTRACT

The lateral flagellum of the antennule of the spiny lobster *Panulirus argus* houses more than 1,000 morphologically similar olfactory sensilla, called aesthetascs. By using a high-resolution activity labeling technique that depends on entry of agmatine into olfactory receptor neurons (ORNs) through cation channels during odor stimulation, we examined the distribution of different functional types of ORNs within and across mature aesthetascs. A significant number of ORNs in mature aesthetascs are labeled with agmatine during stimulation by single odorants, including adenosine-5'-monophosphate, ammonium chloride, cysteine, glycine, proline, and taurine. The percentage of ORNs per aesthetasc that was agmatine labeled during odor stimulation averaged 0.5–1.6% for single compounds and 4.6% for a 33-component mimic of oyster tissue. For most antennules and antennular regions studied, the percentage of agmatine-labeled ORNs by stimulation with single or complex odorants was statistically homogeneous across most or all aesthetascs. The extent of heterogeneity among mature aesthetascs was correlated with their age: extensive heterogeneity was observed only in the distal part of the flagellum containing the oldest aesthetascs and their ORNs. Thus, it appears that over most of the length of the aesthetasc-bearing region of the lateral flagellum, different and distinct functional types of aesthetascs do not exist. Rather, aesthetascs appear to be repetitive morphological and functional units in olfactory coding. However, because odor sensitivity of ORNs can change with the age of an aesthetasc, some development-related functional heterogeneity exists among aesthetascs. *J. Comp. Neurol.* 418:270–280, 2000. © 2000 Wiley-Liss, Inc.

Indexing terms: lobsters; olfactory receptor neurons; agmatine; immunogold-silver techniques

Understanding how the nervous system codes odors requires knowledge of the organization of the peripheral olfactory system, including the distribution of different types of olfactory receptor neurons (ORNs) within the olfactory organ. The nature of such neuronal maps has been examined in a variety of vertebrates. For example, different types of ORNs are distributed randomly throughout the entire olfactory epithelium in fish (Ngai et al., 1993b; Chang and Caprio, 1996) and birds (Leibovici et al., 1996) or within one of several distinct odotopic zones of the olfactory epithelium in amphibians (Mackay-Sim et al., 1982; Freitag et al., 1995) and mammals (Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994; Scott et al., 1997). The goal of the present study was to deter-

mine the distribution of ORN types across the olfactory organ in the spiny lobster, *Panulirus argus*.

The distribution of different types of ORNs has been described in a number of invertebrates, especially arthro-

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pod. The arthropod peripheral olfactory system is compartmentalized into sensilla, and some can be considered a "compound nose" by analogy with the insect compound eye in that they contain many morphologically repeated subunits with similar sensory capabilities (Hekmat-Scafe et al., 1998). In most arthropod species, there exists a variety of morphological types of olfactory sensilla (Altner and Prillinger, 1980). The response spectra of ORNs associated with any one morphological type usually differ from those of ORNs associated with other types of sensilla. However in a single species, morphologically identical sensilla can also house different subsets of ORNs (Kaissling et al., 1989; Steullet and Guerin, 1994; Hansson et al., 1996; Pophof, 1997; Taneja and Guerin, 1997; Clyne et al., 1999; de Bruyne et al., 1999).

Decapod crustaceans possess several types of chemosensilla on their first antennae (antennules). Aesthetasc sensilla, which are located exclusively on the lateral flagellum of the antennules, are considered the prominent type of olfactory sensillum in decapod crustaceans (Hallberg et al., 1992; Fig. 1). Indeed, aesthetascs are the only type of chemosensillum that do not contain mechanoreceptors. Also, aesthetasc ORNs are the only known chemosensory neurons that project into the olfactory lobes, which have a glomerular organization functionally and morphologically analogous to the primary olfactory centers in insects (antennal lobes) and vertebrates (olfactory bulbs; Schmidt and Ache, 1996; Hildebrand and Shepherd, 1997). Each of these sensilla is innervated by up to several hundred ORNs, more than most arthropod olfactory sensilla (Grünert and Ache, 1988; Hallberg et al., 1997). Aesthetasc ORNs respond to food-related odors, including amino acids, ammonium compounds, nucleotides, and organic acids (Derby and Atema, 1988); some also respond to pheromones (Gleeson, 1982; Cate et al., 1999). Aesthetasc ORNs are generally rather narrowly tuned to a few odorants that are not necessarily chemically related (Derby and Atema, 1988). In spiny lobsters, these include taurine-best ORNs, ammonia-best ORNs, adenosine-5'-monophosphate (AMP)-best ORNs, and others (Derby, 2000).

It is not known how aesthetasc ORNs and other crustacean chemoreceptor neurons are distributed across chemosensory organs. Mellon and his collaborators showed that exposure of small groups of aesthetascs to a marker (tritiated leucine) led to labeling of all olfactory glomeruli in the central nervous system (CNS) of the crayfish, *Procambarus clarkii* (Mellon, 1990; Mellon and Munger, 1990). From these results, they argued that ORNs from local groups of aesthetascs, possibly even from individual aesthetascs, have divergent projection patterns and contain a broad diversity of odor-responsive ORNs. Spencer (1986) used multiunit electrophysiological recordings to show that each aesthetasc of the California spiny lobster *Panulirus interruptus* is broadly tuned. Because single-cell recordings were not made, the response spectra of individual ORNs and their distribution across aesthetascs are not known.

The aim of our study was to examine how ORNs with different odorant sensitivities are distributed across the aesthetasc population. By using the Caribbean spiny lobster, *P. argus*, in which each lateral flagellum bears over 1,000 morphologically similar aesthetascs, we asked whether there are different functional classes of aesthetascs based on the sets of ORN types that they contain

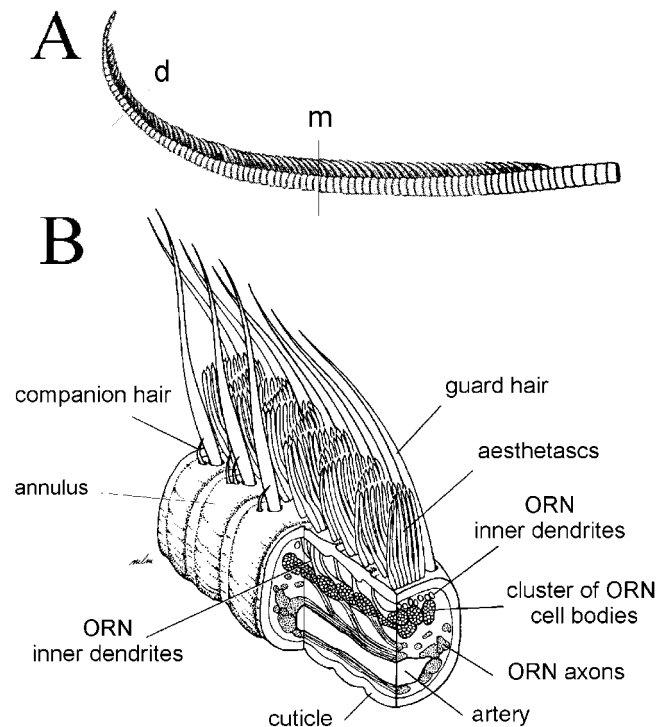


Fig. 1. **A:** Lateral flagellum of the antennule of the spiny lobster. Aesthetascs are located on the distal region of the flagellum. m and d indicate where cross-sections were taken to sample the medial and distal aesthetasc-bearing regions of the lateral flagellum. (Reprinted from Trapido-Rosenthal HG, Carr WES, Gleeson RA. 1989. In: Brand JG, Teeter JH, Cagan RH, Kare MR, editors. Chemical senses, vol I. Receptor events and transduction in taste and olfaction. p 249, by courtesy of Marcel Dekker, Inc.) **B:** Higher magnification view of the aesthetasc-bearing region of a lateral flagellum. This shows that the aesthetascs are organized into two dense rows per annulus. The cell bodies of the olfactory receptor neurons (ORNs) associated with a single aesthetasc are grouped in a cluster and located under the base of the next (proximal) row of aesthetascs. (Reprinted from Michel WC, Steullet P, Cate HS, Burns CJ, Zhainazarov AB, Derby CD. 1999. High-resolution functional labeling of vertebrate and invertebrate olfactory receptor neurons using agmatine, a channel-permeant cation, *J Neurosci Methods* 90:143–156, with permission from Elsevier Science.)

or whether an aesthetasc is both a morphological and functional repetitive unit housing a full range of ORN types. We used a high-resolution odor-dependent labeling technique to localize individual aesthetasc ORNs that are excited by single odorant compounds (AMP, ammonium, cysteine, glycine, proline, and taurine) and by a complex odor mixture (oyster mixture). The activity labeling technique is based on the permeation of agmatine into odor-activated cells (Michel et al., 1999). We limited our analysis to aesthetascs located in the medial and distal part of the lateral flagellum. Most ORNs in these regions are mature and responsive to odors, whereas ORNs in the proximal region of the lateral flagellum are new (Sandeman and Sandeman, 1996; Steullet et al., 1999) and less odor sensitive (Steullet et al., 1999). The results show that each single odorant compound activated a few ORNs in most mature aesthetascs and an average of about 0.5–1.6% of the ORNs in an aesthetasc. The distribution of ORNs responsive to a particular odor was statistically

homogeneous across most or all aesthetascs for most lateral flagella, suggesting that there are no distinct functional types of aesthetascs innervated by different subsets of ORN types. These results show that the aesthetasc constitutes a functional unit in olfactory coding, although some slight development-related variability among aesthetascs exists. Our results tend to support the idea that this olfactory organ functions as a compound nose.

MATERIALS AND METHODS

Animals

Caribbean spiny lobsters (*P. argus*) were collected in the Florida Keys, shipped by air to Georgia State University, held in 800-liter aquaria (20–25°C) containing recirculating, filtered, aerated Instant Ocean (Aquarium Systems, Mentor, OH), and fed shrimp and squid. Preadult animals of 60–75 mm carapace length were used for this study.

Solutions and chemicals

The odor stimuli used were the single chemicals AMP, ammonium chloride (NH₄), cysteine, glycine, proline, and taurine, and an artificial oyster mixture that contains 33 compounds and mimics the composition of oyster extract (Carr and Derby, 1986). All compounds used as odor stimuli and agmatine (1-amino-4-guanidinobutane) were ≥99% pure and were purchased from Sigma (St. Louis, MO), except homarine (a component of the artificial oyster mixture), which was obtained from Chemicals Procurement Laboratories (College Point, NY).

Artificial lobster saline used in activity labeling was composed of (in mM): 458 NaCl, 13.4 KCl, 13.6 CaCl₂, 9.8 MgCl₂, 14.1 Na₂SO₄, 3 HEPES, 1.9 glucose, 1.2 NaOH (pH 7.4). A modified artificial seawater (ASW) used in activity labeling had a low concentration of Na⁺ and Ca²⁺ (10% normal Na⁺ and Ca²⁺) to reduce competition between agmatine and both Na⁺ and Ca²⁺ for entry in the ORNs through cation channels (Michel et al., 1999). The composition of this 10% Na⁺/Ca²⁺ ASW was (in mM): 42 NaCl, 412 N-methyl-D-glucamine-HCl, 9 KCl, 1 CaCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃ (pH 7.2).

For immunocytochemistry, the fixative was prepared daily and contained 2.5% glutaraldehyde, 1% paraformaldehyde, and 10% sucrose in 0.2 M phosphate buffer solution (PBS), pH 7.4. The silver intensification solution was made immediately before use and was composed of 5 ml 0.2 M citrate buffer (pH 4.85), 1 ml 0.3 g/ml hydroquinone, and 1 ml 1% aqueous silver nitrate.

Odor-dependent activity labeling

Odor-dependent activity labeling was accomplished by using agmatine, a channel-permeant cation. Activity labeling with an anti-agmatine antibody was first used on the vertebrate retina (Marc, 1999a,b) and has been adapted to label ORNs of fish and crustaceans (Michel et al., 1999). In lobsters, agmatine penetrates into the ORNs through Na⁺-activated cation channels (Zhainazarov et al., 1998) that are involved in the amplification of the odor-evoked receptor potentials. However, it might also enter the ORNs through other cation channels such as IP₃-gated cation channels (Fadool and Ache, 1992) that are the initial channels activated during odor-mediated excitation (Michel et al., 1999). Therefore, this technique allows labeling of ORNs that are excited, but not inhibited, by an odorant.

To stimulate ORNs, the lateral flagellum of the antennule was first excised and secured in a stimulation chamber (Derby, 1995). The distal part of the flagellum bearing the aesthetascs was placed in an olfactometer with a 9 cm per second flow of 10% Na⁺/Ca²⁺ ASW (see "Solutions and chemicals"). The proximal part of the flagellum was placed in a separate bath containing lobster saline (see "Solutions and chemicals") and dissected to expose the antennular nerve and artery. The antennular artery was cannulated and perfused with oxygenated lobster saline. Odor stimuli were injected for 5 seconds every minute for 60 minutes into the 10% Na⁺/Ca²⁺ ASW flowing over the flagellum, by using an electronically driven valve. Stimuli included 20 mM agmatine (no-odor control) or 20 mM agmatine together with an odor. Odor stimuli were single odorant compounds (see "Solutions and chemicals") presented at 100 μM, and a complex odor mixture, oyster mixture, presented at 1 mM total concentration. After the last odor stimulation, the 10% Na⁺/Ca²⁺ ASW was superfused for another 5 minutes to remove any possible free agmatine before perfusing the antennular artery with fixative (see "Solutions and chemicals"). The flagellum was then removed from the chamber, cut into six equal parts, and placed in fixative for at least 2 days at room temperature. The proximal aesthetasc-bearing region of the flagellum was not used in this study because it contains ORNs that are new and much less responsive to odors (Steullet et al., 1999).

Pieces of the fixed flagellum were rinsed in 0.2 M PBS (pH 7.4), dehydrated with a graded series of ethanol and absolute acetone, infiltrated, and embedded with Pelco Eponate 12 (Ted Pella, Inc., Redding, CA). Transverse sections (0.5 μm thick) were taken from the medial and distal parts of the aesthetasc-bearing region of the flagellum (Fig. 1) by using an ultramicrotome and diamond knife. In the medial and distal parts of the flagellum, at least two rows of aesthetascs were sectioned by taking two serial sections, advancing 10 μm, then taking two more serial sections, advancing another 10 μm, and so forth. This procedure gave an adequate sampling of each cluster of ORN cell bodies associated with each aesthetasc (Fig. 1), because the average diameter of the ORN cell bodies is about 10 μm (Steullet, unpublished data; Laverack and Ardill, 1965). Therefore, whereas two serial 0.5-μm thick sections contain the same ORN cell bodies, each group of two serial sections represents different ORN cell bodies. Indeed, the cell body of a given ORN was typically not found on two groups of serial sections separated by 10 μm.

Individual sections were placed in wells of Teflon-coated spot slides (Erie Scientific, Portsmouth, NH), deplasticized by using sodium ethoxide (saturated sodium hydroxide in ethanol), rinsed in absolute methanol and distilled water, and dried. One of the two serial 0.5-μm thick sections was incubated for 24 hours with a polyclonal anti-agmatine IgG antibody (Chemicon International, Temecula, CA). The other serial section was incubated for 24 hours with a polyclonal anti-aurine IgG antibody (Signature Immunologics, Salt Lake City, UT). The anti-aurine antibody was used to visualize each ORN cell body because it stained the outlines of all ORNs of mature aesthetascs (Fig. 2A), whereas the anti-agmatine antibody did not (Fig. 2B). Furthermore, the anti-aurine antibody allowed us to distinguish between mature and newly differentiated aesthetascs, given that newly differentiated aesthetascs contain ORNs with high cytoplasmic levels of

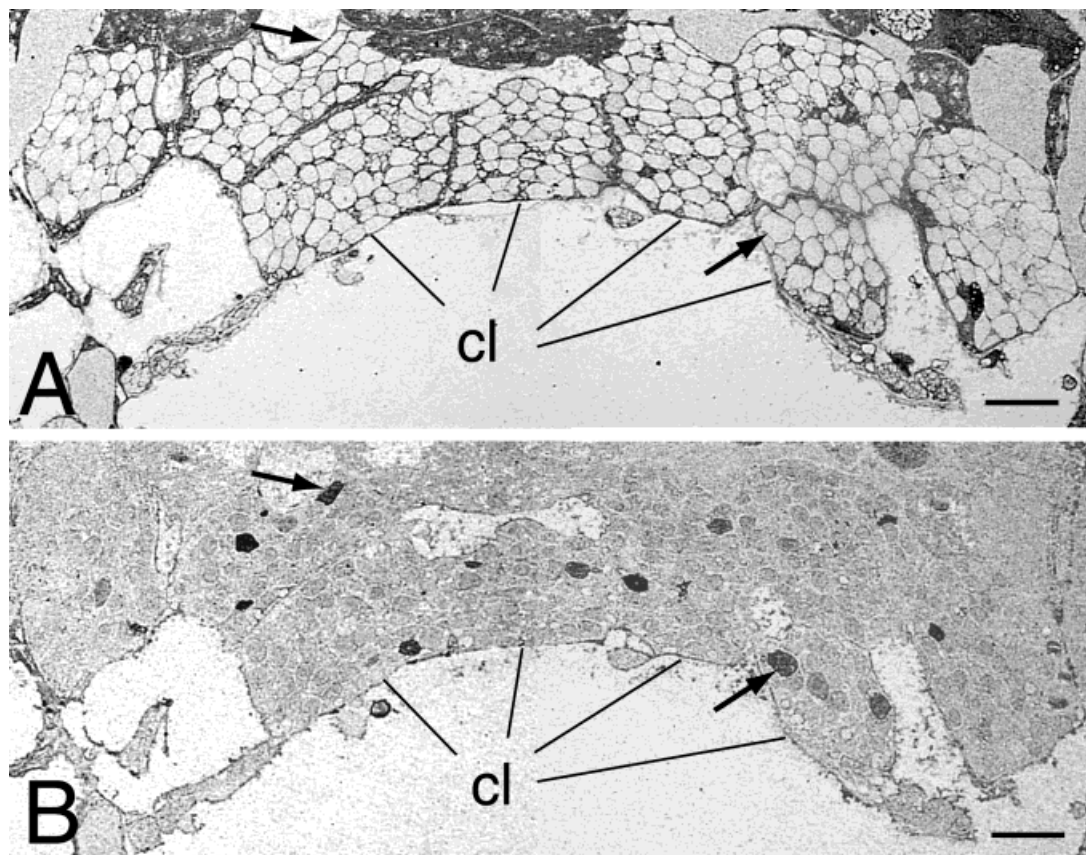


Fig. 2. Transverse cross-sections of a lateral flagellum showing clusters (cl) of cell bodies of olfactory receptor neurons (ORNs) associated with aesthetascs that have been stimulated with 100 μ M proline and 20 mM agmatine. **A:** This section has been treated with an anti-aurine antibody. Note that anti-aurine antibody outlines mature ORNs without staining their cytoplasm. **B:** This section (contig-

uous serial section of A) showing agmatine-like immunoreactivity in the same clusters of ORN cell bodies shown in A. Arrows in both A and B show the position of two agmatine-labeled ORNs, indicating that agmatine entered these cells during odor activation. Scale bars = 25 μ m.

taurine-like immunoreactivity (Steullet et al., 1999). We excluded the newly differentiated aesthetascs from the present study because of their reduced odor sensitivity (Steullet et al., 1999). Agmatine- and taurine-IgG antibodies were raised in rabbits against the glutaraldehyde-conjugated agmatine- and taurine-albumin complex, respectively. They were diluted to 1:1,000 by using 0.1 M PBS (pH 7.4) containing 1% normal goat serum (Amersham, Arlington Heights, IL) and 0.05% thimerosal (Sigma). Sections were rinsed with 0.1 M PBS (pH 7.4) and incubated for 1 hour in a nanogold-conjugated goat anti-rabbit antibody (Amersham) that was diluted 1:100 in a 0.1-M PBS (pH 7.4) containing 1% normal goat serum. The sections were then rinsed with 0.1 M PBS (pH 7.4) and distilled water and exposed to a fresh silver intensification solution (see "Solutions and chemicals") under dark conditions. The silver intensification was stopped after 6 minutes by briefly dipping the sections into 5% acetic acid. Sections were finally rinsed with distilled water and dried.

Quantification of agmatine-labeled cells

Images of the sections were captured by using an Axioskop microscope (Zeiss, Thornwood, NY) with a bright-

field video camera attached to a video board in a computer. Images were analyzed by using the Scion ImagePC software (Scion Corporation, Frederick, MD). Identification of agmatine-labeled ORNs was done by quantifying the agmatine immunoreactivity in the cell bodies of ORNs (Figs. 1, 2).

The number of agmatine-labeled ORNs in each cluster of ORN cell bodies associated with a single aesthetasc (Fig. 1) was calculated by using the anti-agmatine-stained sections. For each cluster, the mean and standard deviation of the pixel intensity of areas that did not contain agmatine-labeled ORNs were used to calculate a 95% confidence interval for background staining. This confidence interval was applied as a cut-off for discriminating agmatine-labeled ORNs from unlabeled ORNs. The total number of labeled ORNs in a single cluster, and thus associated with a single aesthetasc, was the sum of all labeled cells in the given cluster on each 10- μ m-increment anti-agmatine-stained section where this cluster was present. The percentage of agmatine-labeled cells in each cluster of ORN cell bodies (or aesthetasc) was calculated based on an estimation of the total number of ORNs in each cluster. This estimation was done as follows. Each cluster of ORN cell bodies was also individually followed

through the series of 10- μm -increment anti-aurine-stained sections. For each section, the number of ORN cell bodies in a given cluster, and therefore associated with a single aesthetasc, was calculated based on the measured area of this cluster and on the average area occupied by a single ORN in this cluster. The total number of ORNs in the cluster was then the sum of ORNs calculated in the given cluster on each 10- μm -increment anti-aurine-stained section where this cluster was present. An analysis of 304 clusters (from a sample of nine flagella) showed that single aesthetasc bear 292 ± 69 ORNs ($M \pm SD$). Based on these data, we only considered clusters with 179 (lower 95% confidence limit) to 504 ORNs (upper 99.9% confidence limit). A lower 95% confidence limit was chosen to discard clusters that might not have been completely sampled. The upper 99.9% confidence limit was selected to ignore possible outliers that might be the result of an amalgam of indistinguishable neighboring clusters. Indeed, most of the clusters with a number of ORNs above the upper 99.9% confidence limit had unusual shapes, suggesting that they might consist of two neighboring clusters rather than of a single cluster of ORN cell bodies.

RESULTS

The number of ORNs per aesthetasc was 292 ± 69 ($M \pm SD$, $N = 304$ aesthetascs). Stimulation with each of the single odorant compounds typically led to agmatine labeling of at least a few ORNs in each cell body cluster or aesthetasc. An example for proline is shown in Figure 2. Cell bodies of the labeled ORNs in a single cluster were not usually grouped together but were distributed apparently randomly (Fig. 2B).

The percentage of agmatine-labeled cells per aesthetasc was significantly greater for each of the single compounds than for the no-odor control ($P < 0.05$, Kruskal-Wallis nonparametric analysis of variance [ANOVA], Fig. 3). The percentage of agmatine-labeled ORNs for the single odorants ranged from 1.0% for NH_4 to 2.1% for proline, whereas the percentage of agmatine-labeled ORNs for the no-odor control was 0.5%. After subtracting the value for labeling in the no-odor condition, the activity labeling to single odorants can be considered to range from 0.5% for NH_4 to 1.6% for proline. Stimulation with the oyster mixture, which contains 33 compounds, led to agmatine labeling of more ORNs than did the single compounds: an average of 4.6% of ORNs (after subtracting the no-odor labeling). For each of the odorants, the percentage of agmatine-labeled ORNs per aesthetasc did not differ significantly in the distal and the medial parts of the aesthetasc-bearing region of the flagellum ($P < 0.05$, Kruskal-Wallis nonparametric ANOVA, Fig. 3).

To test the hypothesis that an aesthetasc represents a functional and repetitive unit of odor coding, we examined the variability in the percentage of labeled ORNs across aesthetascs. If the aesthetasc is the functional unit, then we would expect the percentage of agmatine-labeled ORNs due to stimulation with a given odorant to be similar from aesthetasc to aesthetasc. The percentage of agmatine-labeled ORNs per aesthetasc ranged from 0 to 6.2% after stimulation with single odorants and 1.3 to 11.6% after stimulation with oyster mixture (no subtraction of no-odor control labeling) (Tables 1, 2). We used the heterogeneity G-test (Sokal and Rohlf, 1997) to examine whether the proportion of labeled ORNs after stimulation

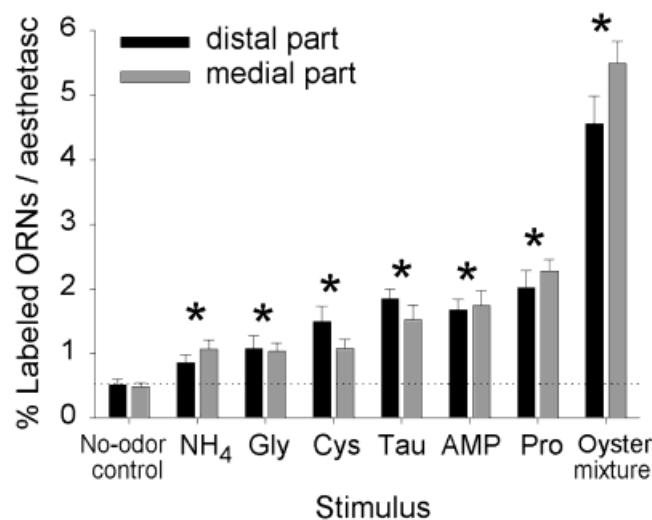


Fig. 3. The percentage of agmatine-labeled olfactory receptor neurons (ORNs) per aesthetasc in the distal (dark bars) and medial (light bars) parts of the aesthetasc-bearing region of a lateral flagellum, after stimulation with either a control (sea water), single odorant compound, or a complex odor mixture (oyster mixture). The values are mean \pm SEM. There is no significant difference in labeling between the medial and distal parts of the flagellum ($P > 0.05$, Kruskal-Wallis analysis of variance [ANOVA]). Stimulation with all single odorant compounds and the oyster mixture causes significantly more agmatine-labeled ORNs than does agmatine alone (no-odor control), as indicated by an asterisk ($P < 0.05$, Kruskal-Wallis ANOVA). The horizontal dotted line shows the level of labeling for the no-odor control. The data were collected from 103 aesthetascs on four flagella for the no-odor control, 53 aesthetascs on two flagella for NH_4 , 40 aesthetascs on two flagella for glycine (Gly), 28 aesthetascs on two flagella for cysteine (Cys), 64 aesthetascs from two flagella for adenosine-5'-monophosphate (AMP), 38 aesthetascs on two flagella for taurine (Tau), 55 aesthetascs on two flagella for proline (Pro), and 73 aesthetascs on three flagella for the oyster mixture.

with a given odorant was homogeneous across aesthetascs of both distal and medial parts of a flagellum. This test assumes that all aesthetascs are replicates and determines if all aesthetascs belong to the same homogeneous set of replicates. Because the heterogeneity G-test is not suitable for data sets for which the expected frequency for each replicate is less than three, we included in this analysis only the 11 flagella that met this requirement of having a minimum of three expected labeled ORNs for each aesthetasc. Three of eight flagella stimulated with a single odorant (flagella 6, 7, and 11 indicated with @ on Tables 1, 2) and two of three flagella stimulated with the oyster mixture (flagella 2 and 3 indicated with @ on Tables 1, 2) showed significant heterogeneity in the proportion of labeled ORNs per aesthetasc ($P < 0.05$, heterogeneity G-test).

For each flagellum, we then examined whether there were regional differences in heterogeneity, by comparing heterogeneity separately in the distal and medial parts of the aesthetasc-bearing region of the flagellum. The distal part of three of eight flagella stimulated with single odorants (flagella 6, 11, 13 in Table 1) and of all three flagella stimulated with the oyster mixture (flagella 1–3 in Table 1) showed significant heterogeneity in the proportion of labeled ORNs per aesthetasc ($P < 0.05$, heterogeneity G-test). In four of these six cases, the significant hetero-

TABLE 1. Degree of heterogeneity in the proportion of labeled olfactory receptor neurons (ORNs) per aesthetasc in the distal part of the flagellum¹

Flagellum no.	Odorant	No. of aesthetascs analyzed	Range of % labeled cells per aesthetasc	<i>P</i> value from heterogeneity G-test	No. aesthetascs responsible for the heterogeneity
1	Oyster	4	5.6–11.6	0.028*	2
2@	Oyster	16	1.3–7.7	0.048*	2
3@	Oyster	14	1.4–8.0	0.0000001*	10
4	NH ₄	14	0.0–1.4	—	—
5	NH ₄	14	0.0–2.7	—	—
6@	AMP	13	0.0–4.1	0.036*	2
7@	AMP	15	0.5–2.6	NS 0.696	—
8	Cysteine	11	0.0–3.2	NS 0.209	—
9	Taurine	10	1.0–2.4	NS 0.982	—
10	Taurine	11	0.9–3.8	NS 0.353	—
11@	Proline	14	0.0–6.2	0.000001*	8
12	Proline	17	0.0–4.3	NS 0.159	—
13	Glycine	15	0.4–4.3	0.029*	2
14	Glycine	17	0.0–1.1	—	—
15	No-odor control	6	0.0–2.3	—	—
16	No-odor control	16	0.0–1.6	—	—
17	No-odor control	16	0.0–1.5	—	—
18	No-odor control	12	0.0–0.9	—	—

¹Each flagellum was stimulated with one odorant. Heterogeneity among the indicated number of aesthetascs was tested for each flagellum using a heterogeneity G-test (Sokal and Rohlf, 1997). An asterisk and bold lettering mark cases of significant heterogeneity ($P < 0.05$). No *P* value indicates that the flagellum did not have enough labeled ORNs per aesthetasc to be tested for heterogeneity (i.e., less than three expected ORNs per aesthetasc). For the six flagella with significant heterogeneity, the right-hand column shows the number of aesthetascs responsible for the heterogeneity. The range of percent labeled cells given is without subtraction of an average labeling for the no-odor control. @ indicates flagella with significant heterogeneity when aesthetascs from both medial and distal regions were analyzed together. AMP, adenosine-5'-monophosphate.

TABLE 2. Degree of heterogeneity in the proportion of labeled olfactory receptor neurons (ORNs) per aesthetasc in the medial part of the flagellum¹

Flagellum no.	Odorant	No. of aesthetascs analyzed	Range of percent labeled cells per aesthetasc	<i>P</i> value from heterogeneity G-test	No. aesthetascs responsible for the heterogeneity
1	Oyster	5	6.7–11.6	NS 0.319	—
2@	Oyster	17	2.5–6.9	NS 0.338	—
3@	Oyster	17	2.0–9.2	0.029*	2
4	NH ₄	9	0.3–2.6	—	—
5	NH ₄	20	0.0–3.2	—	—
6@	AMP	18	0.0–2.2	—	—
7@	AMP	19	0.7–6.0	0.019*	2
8	Cysteine	17	0.0–2.2	—	—
9	Taurine	8	0.8–3.5	NS 0.291	—
10	Taurine	9	0.0–1.8	—	—
11@	Proline	9	2.0–4.1	NS 0.681	—
12	Proline	12	1.0–2.9	NS 0.861	—
13	Glycine	22	0.4–2.7	NS 0.612	—
14	Glycine	20	0.0–1.1	—	—
15	No-odor control	8	0.4–1.7	—	—
16	No-odor control	13	0.0–0.9	—	—
17	No-odor control	15	0.0–1.3	—	—
18	No-odor control	17	0.0–0.9	—	—

¹Each flagellum was stimulated with one odorant. Heterogeneity among the indicated number of aesthetascs was tested for each flagellum using a heterogeneity G-test (Sokal and Rohlf, 1997). An asterisk and bold lettering mark cases of significant heterogeneity ($P < 0.05$). No *P* value indicates that the flagellum did not have enough labeled ORNs per aesthetasc to be tested for heterogeneity (i.e., less than three expected ORNs per aesthetasc). For the two flagella with significant heterogeneity, the right-hand column shows the number of aesthetascs responsible for the heterogeneity. The range of percent labeled cells given is without subtraction of an average labeling for the no-odor control. @ indicates flagella with significant heterogeneity when aesthetascs from both medial and distal regions were analyzed together. AMP, adenosine-5'-monophosphate.

geny resulted only from the two aesthetascs with the highest and lowest proportion of labeled ORNs, whereas all the other aesthetascs together formed a homogeneous set (unplanned comparisons between aesthetascs by using a simultaneous test procedure; Sokal and Rohlf, 1997; see Table 1). However, in the distal region of two flagella (flagella 3 and 11 in Table 1), many aesthetascs contributed to the significant heterogeneity. This heterogeneity was due in part to the presence of a large proportion of aesthetascs that contained no or very few labeled ORNs in the distal region of the flagellum, whereas the highest percent labeling observed in aesthetascs remained quite similar in both the distal and medial regions (Fig. 4).

In contrast to the distal part, the medial part of only two of eight flagella (flagella 3 and 7 in Table 2) showed significant heterogeneity across aesthetascs ($P < 0.05$, heterogeneity G-test). In both cases, only the two aesthetascs with the lowest and highest labeling contributed to this

heterogeneity, whereas all the other aesthetascs formed a homogeneous set (unplanned comparisons between aesthetascs by using a simultaneous test procedure; Sokal and Rohlf, 1997; see Table 2). The lower probability of significant heterogeneity in the medial vs. distal part of the flagellum appeared to be due to the presence of fewer aesthetascs with no or low-activity labeling in the medial part (Fig. 4).

There was no relationship between the likelihood of an aesthetasc to contribute to the heterogeneity among a group of aesthetascs (Tables 1, 2) and that aesthetasc's position within a row. The aesthetascs that were responsible for the heterogeneity were equally likely to be in the mesial, central, or lateral positions in the row. Finally, the percentage of aesthetascs with no agmatine labeling per flagellum was inversely correlated with its average agmatine labeling per aesthetasc (excluding aesthetascs with no labeling) ($P < 0.05$, $r^2 = -0.52$, $N = 18$, Pearson

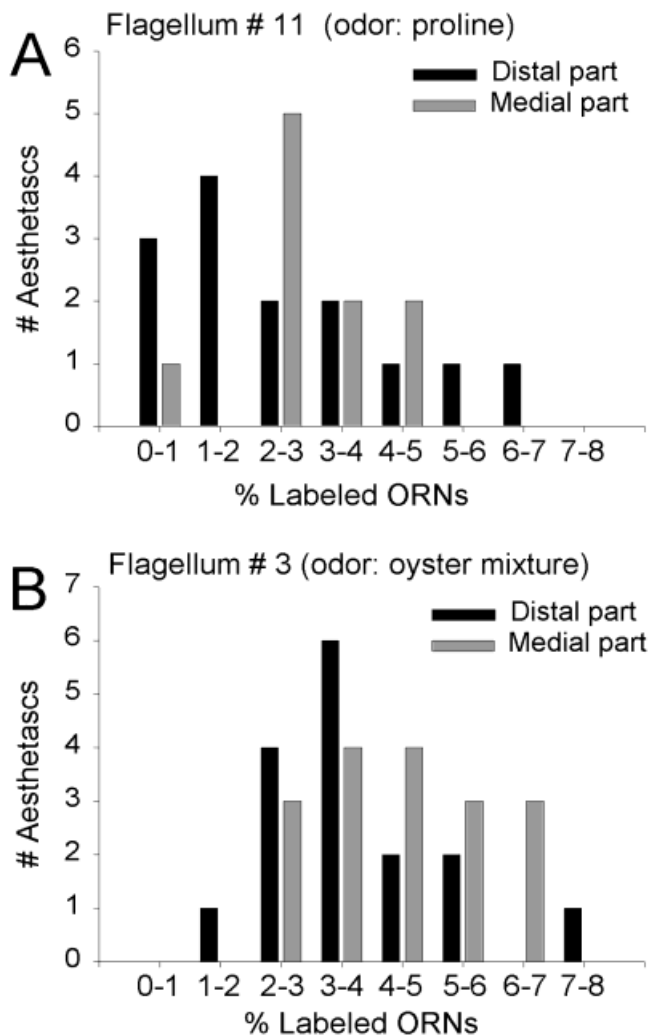


Fig. 4. Distribution of aesthetascs based on percentage of agmatine-labeled olfactory receptor neurons (ORNs) in both distal and medial parts of the flagellum. The two flagella illustrated here (A: flagellum 11, stimulated with proline; B: flagellum 3, stimulated with the oyster mixture) show a large degree of heterogeneity among aesthetascs in the distal part (see Table 1) but not in the medial part of the flagellum (see Table 2). Note the larger proportion of aesthetascs with low-activity labeling in the distal vs. medial part of the flagellum, whereas the maximum percent labeled cells per aesthetasc are similar in the distal and medial parts.

product-moment correlation, Fig. 5). This suggests that aesthetascs with no labeling did not reveal the presence of a functionally distinct type of aesthetascs, but rather simply shows that the less stimulatory an odorant is, the less likely it is that an aesthetasc will contain odor-responsive ORNs.

DISCUSSION

We used high-resolution odorant-dependent activity labeling with the channel-permeant cation agmatine to characterize the distribution of ORNs in mature aesthetasc sensilla on the lateral flagella of the spiny lobster, *P. argus*. The total number of ORNs innervating a single

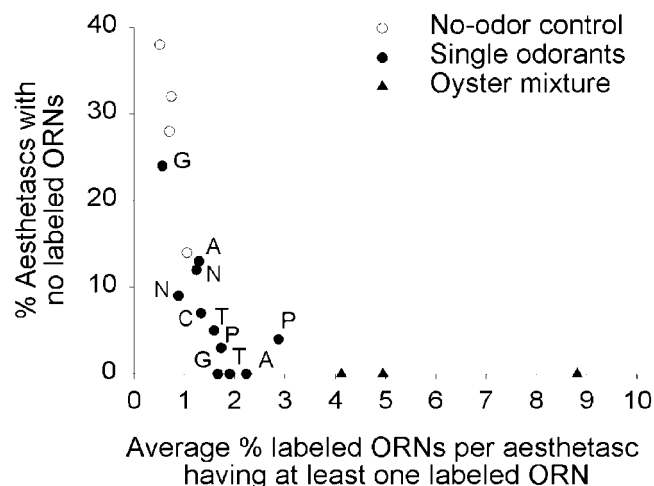


Fig. 5. The percentage of aesthetascs with no agmatine-labeled olfactory receptor neurons (ORNs) is inversely correlated with the average percentage of agmatine-labeled ORNs per aesthetasc with at least one labeled ORN. Each point represents the average value for all aesthetascs examined in a single flagellum. This graph shows that the less the stimulatory an odorant is, the less likely it is that an aesthetasc will contain odor-responsive ORNs. This result supports the idea that aesthetascs without any agmatine-labeled ORNs are not functionally distinct types of aesthetascs. Single odorants: adenosine-5'-monophosphate (A), cysteine (C), glycine (G), NH_4 (N), proline (P), and taurine (T).

aesthetasc averages approximately 300, but ranges from less than 200 to more than 500. After subtracting the level of agmatine labeling induced by stimulation with agmatine alone (i.e., the no-odor control condition), single odorant compounds effectively activity labeled about 0.5–1.6% of the ORNs of mature aesthetascs; a 33-component mimic of oyster tissue effectively activity labeled 4.6% of the ORNs. The percentage of agmatine-labeled ORNs due to stimulation with each of the odorants tested was statistically homogeneous across most or all aesthetascs on most lateral flagella. Furthermore, homogeneity was most common in the medial part of the flagellum, whereas extensive heterogeneity was sometimes found in the distal part containing the oldest aesthetascs.

Agmatine-labeled ORNs represent cells best tuned to the odor stimulus

Agmatine penetrates into lobster ORNs through Na^+ -activated nonselective cation channels (Zhainazarov et al., 1998; Michel et al., 1999) that are involved in the amplification during odor transduction. It might also enter through other cation channels such as IP_3 -gated cation channels (Fadool and Ache, 1992) that are the primary source for odor-mediated excitation. Based on the sensitivity of this odor-dependent activity labeling technique and on the physiological properties of lobster ORNs, we believe that only ORNs that are excited by their best stimulatory odorants are labeled. Indeed, electrophysiological analyses of response profiles of ORNs to sets of single odorant compounds that include those used in the present study showed that spiny lobster ORNs are rather narrowly tuned (Derby et al., 1991; Cromarty and Derby, 1997; Steullet and Derby, 1997; Derby, 2000). One odorant

compound is usually a much more effective excitant than any other compound. Thus, there are taurine-best cells, AMP-best cells, cysteine-best cells, NH_4 -best cells, and so forth. Other compounds besides the "best" odorant can excite an ORN, but the "next-best" odorant must be at a very high concentration to be as excitatory as the best compound. For example, for taurine-best cells, 10^{-8} M taurine evokes half-maximal responses, whereas the concentration of next-best compounds required to evoke half-maximal responses is on average five orders of magnitude greater (Cromarty and Derby, 1997). On the other hand, significant numbers of aesthetasc ORNs are agmatine labeled only by stimulation with concentrations of 10 μM taurine or greater (Michel et al., 1999). This suggests that this odor-dependent activity labeling technique has a low sensitivity and can only label cells that are strongly excited. Together, these data support the idea that, at the odorant concentration used in this study, stimulation with each single odorant results in activity labeling of ORNs with best tuning to it and not any of the other ORNs, even those for which that odorant is a next-best compound. So, stimulation with 100 μM taurine activity labels only taurine-best ORNs, stimulation with 100 μM AMP activity labels only AMP-best cells, and so on. Therefore, we believe that agmatine following stimulation by a given odorant provides a good estimate of percentage of ORNs best-tuned to that odorant, but it is likely an underestimate of the number of ORNs that are responsive but not best-tuned to that odorant.

Stimulation with oyster mixture at 1 mM activity labeled an average of 4.6% of ORNs, following correction for labeling by stimulation with the no-odor control. This is an expected level of labeling. Indeed 1 mM of the 33-component mixture, oyster mixture, contains only two compounds that are >100 μM and only 10 compounds >10 μM . Given that levels of activity labeling are significantly above controls only if single odorant compounds are presented at 10 μM or greater (Michel et al., 1999) and that stimulation by each single odorant compound at 100 μM activity labeled 0.5–1.6% of the ORNs, a 4.6% labeling following stimulation by the oyster mixture is not unexpected, even if the percentage of labeled cells by the compounds is additive.

Single odorants activate small subsets of ORNs

If the number of ORNs agmatine labeled by stimulation with a single odorant is a good representation of the number of ORNs best tuned to that odorant as argued in the above section, then ORNs best tuned to a single odorant constitute only a small proportion of the entire population of ORNs (about 0.5–1.6%). Indeed, a general feature of most nonpheromonal olfactory systems is that a single odorant only activates a small fraction of the entire population of ORNs. Conversely, each olfactory system has a large variety of ORNs. Each odorant is believed to be detected by more than one type of receptor molecule (Krautwurst et al., 1998) and each type of odor receptor molecule is usually expressed in a very small fraction of ORNs (0.1–0.2% of ORNs in rodents: Ressler et al., 1993; 0.5–2.5% in fish: Ngai et al., 1993a; Barth et al., 1996). It is not surprising, then, that single odorants activate only a few percent of the ORNs in mammals (Restrepo et al., 1993; Rawson et al., 1997) and 2.5–20% of the ORNs in zebrafish (Michel et al., 1999). Information concerning the

proportion of ORNs that respond to single odorants is still quite limited for invertebrates. In addition to our results, the available data in other invertebrates suggest that single odorants activate small subsets of ORNs. In the tick, *Amblyomma variegatum*, which possesses a limited number of olfactory sensilla (19) and ORNs (about 90) on the tarsus of the anterior leg, each single identified semiochemical activates only two to three ORNs (Steullet and Guerin, 1994). In *Drosophila*, about 35 classes of ORNs, based on their odor specificity, have been described (Clyne et al., 1999; de Bruyne et al., 1999). In situ hybridization shows that a single odorant receptor is expressed in about 0.1–1.5% of the entire population of ORNs (Clyne et al., 1999; Vosshall et al., 1999). The olfactory system of the pine weevil (Coleoptera) contains at least 30 types of ORNs responding to plant odors (Wibe et al., 1997). Thus, despite great differences in complexity and structure of the olfactory systems across the animal kingdom, the available data suggest that, in general, peripheral olfactory systems of vertebrates and invertebrates have a diversity of ORN types that enable animals to capture the complexity of the chemical signals in both aquatic and terrestrial environments.

No distinct functional classes of aesthetascs

The proportion of activity-labeled ORNs per aesthetasc in *P. argus* varies to some extent, but averages 0.5–1.6% for single odorant compounds. Most aesthetascs contain at least a few ORNs that respond to a given single odorant. Furthermore, this relationship holds true for aesthetascs in the distal and medial parts of the lateral flagellum. These results argue against the existence of different functional types of aesthetascs in most of the flagellum, and against functional heterogeneity of the aesthetascs on a flagellum. Whereas homogeneity in aesthetasc response profiles is the general rule, there does appear to be more heterogeneity in the distal than in the medial part of the flagellum. In our study, there was an especially large degree of heterogeneity in the distal part of 2 of the 11 flagella examined (Table 1). In both of these flagella, the proportion of aesthetascs with no or only a very small fraction of labeled ORNs was greater in the distal than in the medial part of the flagellum (Fig. 4). Because a loss of aesthetascs occurs during each molt in the distal part of the aesthetasc region (Steullet et al., 1999), this may reflect a loss or at least a reduction of odor sensitivity of ORNs in the distally located aesthetascs that will disappear at the next molt. On both medial and distal parts of the flagellum, the aesthetascs that have the lowest or highest percentages of labeled ORNs are not located in consistent and well-defined positions within rows of aesthetascs. Because the proportion of aesthetascs with no labeled ORNs in a flagellum appears to be inversely correlated with the average percentage of labeling observed in aesthetascs having labeled ORNs, this suggests that aesthetascs with no labeled ORNs do not constitute a functionally distinct type of aesthetascs. This and the fact that heterogeneity was observed only in a fraction of the flagella examined support the idea of an absence of fixed patterns of functional classes of aesthetascs within rows and along the flagellum.

Our results suggest that there do not exist any distinct functional types of aesthetascs. Rather, the aesthetascs are generally homogenous in their function, with the limited heterogeneity in odor sensitivity being partly related

to the aesthetasc age and possibly to damage of the sensilla. Aesthetascs also had no fixed number of ORNs, which can influence the number of labeled ORNs in each aesthetasc. The reasons for the variability in the total number of ORNs per aesthetasc are unknown. One possible reason is a technical one. We determined the total number of ORNs in each aesthetasc from a sampling of each cluster of ORN cell bodies by using 10- μ m-increment sections and from a calculation of the total number of ORNs in each cluster by using an average diameter for a single ORN. However, we do not believe that our quantification method is responsible for the observed variation in the number of ORNs per aesthetasc. This is because our estimated range of ORN number per aesthetasc (292 ± 69 ORNs per aesthetasc; $M \pm SD$, $N = 304$) was quite similar to that reported previously in *P. argus* (Laverack and Ardill, 1965; Grünert and Ache, 1988) and *P. interruptus* (Spencer and Linberg, 1986). For instance, Spencer and Lindberg (1986) found that aesthetascs in *P. interruptus* contain 262 ± 52 ORNs ($M \pm SD$) by using a different technique than ours, i.e., counting the number of inner dendrites associated with an aesthetasc (one inner dendrite per ORN). Thus, we believe that most of the variability observed in the total number of ORNs and the proportion of labeled ORNs per aesthetasc cannot be attributed to our quantification method, but rather to properties of the system.

Several biological phenomena might account for the observed variability in the total number of ORNs per aesthetasc. In adult rodents, many differentiating ORNs eventually die after a few weeks, presumably because their axons fail to make proper connections in the olfactory bulbs (Mackay-Sim and Kittel, 1991). Similarly, in lobsters, a fraction of the densely packed aesthetasc ORNs may die after failing to reach and form functional synapses in the olfactory lobes, which are more than 10 cm away from the aesthetasc ORN somata. This and other factors, such as the number of programmed cell divisions and cell deaths during sensillar development (Ray and Rodrigues, 1995), may vary across aesthetascs and account for the variability in total number of ORNs per aesthetasc. Time available for differentiation of the aesthetascs might be a limiting factor. Indeed, whereas new aesthetascs are emerging at the cuticular surface at the flagellum at each molt (Steullet et al., 1999), the initiation of stem cell differentiation and division during the intermolt stage that leads to the formation of these new aesthetascs is not synchronized. There is a spatiotemporal wave of development of the aesthetascs (Steullet, unpublished data). Thus, the period during which a sensillum differentiates until the molt differs; this may lead to some variability in the total number of ORNs per aesthetasc and, subsequently, in the number of a given functional type of ORNs per aesthetasc. Finally, some stochastic mechanisms might also be involved in the expression of odorant receptors, because the number of agmatine-labeled ORNs per aesthetasc after stimulation with a given odorant is not significantly different from a Poisson distribution (Steullet, unpublished data). However, the number of aesthetascs that we analyzed in each flagellum was not large enough to exclude other mechanisms. The ORNs agmatine labeled by a stimulation with the same single odorant were randomly distributed within an aesthetasc, supporting the idea that the expression of the odor specificity is a relatively late event during the devel-

opment of the sensillum, as observed in the olfactory epithelium of vertebrates (Ressler et al., 1993).

Our results on spiny lobsters and those of Mellon and colleagues on crayfish (Mellon, 1990; Mellon and Munger, 1990) show, respectively, that most or all aesthetascs contain a similar and high diversity of ORN types and that the ORNs from a small group of aesthetascs project into all glomeruli of the olfactory lobe. These observations together with the electrophysiological data of Spencer (1986) strongly suggest that an aesthetasc is a functional and morphological repetitive unit of the olfactory system in decapod crustaceans. This contrasts with the organization of the olfactory system in some other arthropods, in which morphologically identical olfactory sensilla can be innervated by different and distinct subsets of ORNs (fruit flies: de Bruyne et al., 1999; moths: Pophof, 1997; locusts: Hansson et al., 1996; bugs: Taneja and Guerin, 1997; ticks: Steullet and Guerin, 1994). The spatial arrangement and dense distribution of a repetitive, functional unit—the aesthetasc—along the lateral flagellum may be an adaptation to sampling in an environment where odor plumes are typically highly discontinuous and contain many small odor filaments or packets (Moore and Atema, 1991; Atema, 1995). The antennules of *P. argus* are long, articulated, flexible, and highly mobile. Consequently, they can sample a space as large as a few hundred cubic centimeters. Moreover, because the aesthetasc region on the lateral flagellum of *P. argus* can be as long as 3 cm, only one or a few separate regions of the aesthetasc-bearing part of the lateral flagellum might be stimulated at a given time by an odor plume. Consequently, having an olfactory organ made of many replicates of a single functional and structural unit might provide spiny lobsters with sensory inputs about the quality and intensity of an odor packet that would be relatively invariable and independent of the location of the aesthetasc region that was stimulated.

Another possible advantage of the repetitive, functional unit organization of the lobster olfactory system may also be related to development and growth. Lobsters grow indefinitely through multiple molts, during which occurs a massive turnover in the peripheral olfactory system that includes both loss and addition of aesthetascs (Steullet et al., 1999). Therefore, having an olfactory organ composed of a repetitive unit would certainly simplify the developmental processes leading to a continual reorganization of the olfactory organ.

In conclusion, aesthetascs are usually innervated by a similar diversity of types of ORNs. Consequently, the aesthetascs cannot be classified into discrete functional classes. There is a certain level of variability in the response profiles of aesthetascs, some of which is related to a developmental axis along the flagellum. These results suggest that an aesthetasc may be considered as a repetitive morphological and functional unit with some minor variations on a theme, and that the crustacean lateral flagellum is functionally organized as a compound nose.

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