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PERIRECEPTOR MECHANISMS SUSTAINING OLFACTION AT LOW SALINITIES: INSIGHT FROM THE EURYHALINE BLUE CRAB *CALLINECTES SAPIDUS*

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Summary

As the blue crab *Callinectes sapidus* moves from sea water to fresh water, the 'exposed' chemosensory dendrites in the olfactory sensilla (aesthetascs) undergo changes in length that are positively correlated with environmental salinity. In this study, we demonstrate the following. (1) The responses of the olfactory receptor cells of freshwater-acclimated crabs are reduced relative to those of seawater-acclimated animals, but increase with a time course comparable to the increase in dendrite length when these crabs are transferred to sea water. (2) The olfactory response of seawater-acclimated crabs is lost and the chemosensory dendrites osmotically ablated if the aesthetascs are acutely exposed to low salinity. However, maintaining iso-osmotic conditions with mannitol preserves both the physiological response and the structural integrity of the dendrites. (3) The flux of [^{14}C]thiocyanate and ^{22}Na between the hemolymph and sensillar lymph of the aesthetascs indicates continuity between these fluid compartments. (4) There is a net efflux of Na^+ from the hemolymph through the aesthetascs in freshwater-acclimated crabs, and measurements of electrical potential across the antennules suggest that this efflux largely derives from passive diffusion. (5) Dendrites in the aesthetascs of crabs acclimated to brackish water are intermediate in length between those of freshwater- and seawater-acclimated animals. Together, our findings suggest that, at low salinities, the efflux of Na^+ (and probably other ions) from the hemolymph generates an ionic/osmotic microenvironment within the aesthetascs that sustains the structural and functional integrity of the olfactory dendrites. We propose that the length of these dendrites, and consequently the olfactory response, is constrained by the distance over which this microenvironment can be effectively maintained.

Key words: blue crab, *Callinectes sapidus*, olfaction, salinity, aesthetasc, dendrites, perireceptor mechanisms, osmotic effects.

Introduction

A critically important aspect of olfaction is that of providing access of the odors to receptors such that adequate quantitative, qualitative and temporal resolution of the odor environment can be made. Allowing this access necessarily requires 'exposing' the dendrites of olfactory receptor cells to the external environment. For aquatic organisms inhabiting waters in which the ion concentrations are low and/or variable, such exposure of the dendrites must be balanced by mechanisms providing the appropriate ionic and osmotic transmembrane gradients necessary to sustain neural function. Although the relationship between olfactory responses and the ionic composition of the extracellular milieu has been examined in a number of studies on aquatic organisms (e.g. Tucker and Shibuya, 1965; Suzuki, 1978; Leveteau *et al.* 1989; Schmiedel-Jakob *et al.* 1990), there are few data on how altered salinity *per se* affects olfactory systems and essentially no information on perireceptor mechanisms (Getchell *et al.* 1984; Carr *et al.* 1990) that might sustain olfaction in ionically and osmotically challenging salinity conditions.

We previously showed that the olfactory sensilla (aesthetascs) of the euryhaline blue crab *Callinectes sapidus* contain the finely branched outer dendritic segments (ODSs) of between 40 and 160 olfactory receptor cells; as in the aesthetascs of other crustaceans (e.g. Heimann, 1984; Grünert and Ache, 1988), the 'exposed' portions of these dendritic processes are bathed by a sensillar lymph and are separated from the external environment only by a thin odor-permeable cuticle (Gleeson *et al.* 1996). A discovery of particular importance in that study was that the length of the 'exposed' ODSs (i.e. the part extending beyond the constricted region in the aesthetasc) varies as a function of salinity. The length of the ODSs in freshwater (FW)-acclimated blue crabs is substantially shorter than that measured in crabs acclimated to sea water (SW). When FW-acclimated blue crabs are

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transferred to SW, there is a rapid increase in ODS length, with the length approaching that of SW-acclimated animals by 4 days of exposure; conversely, sequential transfer of SW-acclimated crabs to lower salinities brings about rapid reductions in ODS length (Gleeson *et al.* 1996).

The distribution of the blue crab ranges from hypersaline lagoons (salinity 60–80‰; Simmons, 1957) to freshwater rivers and lakes (salinity <0.5‰; Gunter, 1938; Mangum and Amende, 1972). As it moves into waters of lower salinity, specialized epithelia associated with the posterior gills actively transport Na^+ and Cl^- from the surrounding medium into the hemolymph, allowing the crab to regulate the ionic concentration of its extracellular fluid significantly above ambient levels (Mantel, 1967; Copeland and Fitzjarrel, 1968; Ballard and Abbott, 1969; Smith and Linton, 1971; Tagatz, 1971; Lynch *et al.* 1973; Colvocoresses *et al.* 1974; Towle *et al.* 1976; Engel, 1977; Cameron, 1978; Neufeld *et al.* 1980; Aldridge and Cameron, 1982; Gilles and Pequeux, 1986; Pequeux *et al.* 1988; Burnett and Towle, 1990). At low salinities, therefore, the cell bodies of the olfactory receptor cells innervating the aesthetascs are bathed in a hemolymph having ionic and osmotic concentrations markedly above those of the external milieu. Assuming that the cuticle of the blue crab's aesthetasc is freely permeable to ions, as suggested by studies of lobster aesthetascs (Gleeson *et al.* 1993), it is important to consider what might be the composition of the fluid (i.e. sensillar lymph) immediately surrounding the exposed ODSs. Are there mechanisms, for example, that regulate this fluid compartment so that the ionic/osmotic concentrations approximate those of the hemolymph that bathes the remainder of the olfactory receptor cell? Alternatively, does the composition of the sensillar lymph more closely reflect that of the external medium, necessitating the involvement of other compensatory mechanisms to maintain the structural and functional integrity of the exposed dendrites?

In the present study, using neurophysiological techniques, we examine the olfactory responses of crabs acclimated to (or briefly exposed to) different salinities and, using radiotracer techniques, explore the ion-flux relationships between the hemolymph and sensillar lymph of the aesthetascs. Our results, together with new and previously reported morphological findings, reveal the challenges to olfaction in varying ionic/osmotic conditions and suggest a mechanism by which olfactory function is sustained at low salinities.

Materials and methods

Animals

SW-acclimated male specimens of *Callinectes sapidus* Rathbun in intermolt condition were obtained from commercial fishermen trapping in the tidal creeks of local salt marshes. These animals were held at the Whitney Laboratory in tanks with a flow-through supply of ambient sea water (temperature range 15–28 °C). FW-acclimated males in intermolt were procured from commercial crab fishermen trapping on the western shore of Lake George, a large freshwater segment of the St Johns River. These crabs were held at the Whitney Laboratory in tanks containing aerated water (25 °C) obtained from a deep well near Lake George or water collected directly from Lake George. Concentrations (in $mmol\,l^{-1}$) of the major components for the well water and water from Lake George were: (well) 4.40 Na^+, 3.44 Cl^-, 0.74 Ca^{2+}, 0.26 Mg^{2+}, 0.06 K^+; (Lake George) 6.74 Na^+, 8.37 Cl^-, 1.26 Ca^{2+}, 0.96 Mg^{2+}, 0.17 K^+. All animals were sustained on a diet of shrimp, fish and squid.

Neurophysiological procedures

Extracellular, multiunit recordings were made from axon bundles innervating the aesthetascs utilizing a procedure similar to that employed for lobster antennules (Gleeson and Ache, 1985). The excised antennule was placed in a recording chamber/olfactometer (Fig. 1) and sustained by arterially

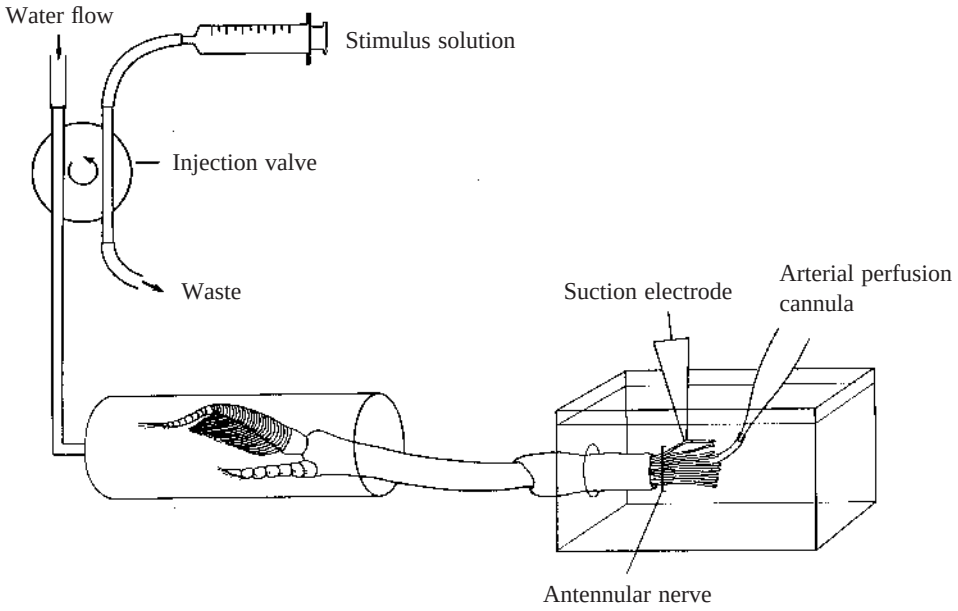

Fig. 1. Schematic diagram of the recording chamber/olfactometer used in monitoring the extracellular activity of olfactory receptor cells in blue crab (*Callinectes sapidus*) antennules.

perfusing the preparation with an oxygenated saline solution. A carrier stream of water, either artificial sea water (ASW) (see Gleeson *et al.* 1989) or well water, into which 190 µl samples of an odor stimulus could be introduced, continuously flowed past the tip of the antennule at a rate of 3 ml min^{-1}. Using a suction electrode, *en passant* recordings of evoked action potentials were made from axon bundles dissected from the antennular nerve.

Two different perfusion salines were employed. One described by Mulloney and Selverston (1974) and routinely used in electrophysiological studies on the spiny lobster (*Panulirus argus*) was utilized as a perfusion medium for all crabs maintained in SW; this is referred to as 'SW-saline'. Another saline (FW-saline) was developed for perfusing the antennules of FW-acclimated crabs; this was based on measurements of hemolymph electrolyte concentrations in blue crabs from Lake George [hemolymph values (in mmol l^{-1}) for eight animals were: 429±12 Na^+, 296±2 Cl^-, 10.3±0.4 Ca^{2+}, 3.15±0.14 Mg^{2+}, 4.60±0.14 K^+, mean ± S.E.M.]. The FW-saline contained (in mmol l^{-1}): 159 sodium gluconate, 270 NaCl, 4.6 KCl, 10.3 $CaCl_2$, 3.2 $MgSO_4$, 1.7 Hepes, 0.94 glucose, pH adjusted to 7.4.

An extract of TetraMarin Flake Food (TetraWerke, Germany), a commercial fish food, was used as the chemical (odor) stimulus in all experiments. A stock solution, identical to that used for olfactory studies in *Panulirus argus* (e.g. Michel and Ache, 1992), was prepared by stirring 3.3 g of TetraMarin into 100 ml of SW-saline for 3–4 h, centrifuging the slurry to remove larger particles, then filtering the supernatant through Whatman size 2 filter paper. The filtrate was then divided into samples and frozen until use. For each experiment, a 500-fold dilution of the TetraMarin stock solution was utilized as the 'standard' odor stimulus; concentration–response data were obtained using log-step dilutions of this standard. All dilutions were made from the same batch of water used for the carrier medium of the olfactometer.

Previous work showed that the antennular nerve of the blue crab consists of three major bundles (Gleeson, 1978). Two of these are largely formed by the axons from olfactory receptor cells innervating the aesthetascs on the lateral and medial portions of the outer flagellum; the third contains axons derived from both chemo- and mechanosensory cells associated with the inner flagellum. This latter bundle is easily distinguished physiologically from the others by characteristically high levels of spontaneous activity in many of the chemo- and mechanosensory cells. In these studies, all recordings were obtained from the two bundles containing the axons of olfactory receptor cells.

It was generally not possible to resolve the typically low-amplitude action potentials of single cells in these recordings; therefore, multiunit activity was monitored. This was accomplished using a voltage-amplitude window discriminator. The lower threshold of the window discriminator was adjusted such that events exceeding the maximum amplitude of the background activity were counted. At the beginning of each experiment, a water blank (the same water used to dilute the TetraMarin) was introduced into the carrier flow of the olfactometer to test for any change in activity due to the diluent itself. If the water blank had little or no effect, TetraMarin odor stimuli were then introduced. With each odor stimulus presentation, a 10 s count of the impulses generated was made from the time of stimulus arrival at the aesthetascs. Any background activity exceeding threshold was measured for 10 s intervals immediately prior to stimulation and was subtracted from the 'odor stimulus' count to yield the olfactory response. A minimum interval of 2 min was maintained between stimulus presentations.

The effect of acute salinity stress on the olfactory response was examined in experiments in which the aesthetascs of SW-acclimated crabs were exposed to 10 % ASW with and without added mannitol (0.82 mol l^{-1}) to maintain iso-osmotic conditions. In these experiments, the olfactory response was examined sequentially using the following paradigm: (1) the response was measured in 100 % ASW; (2) the carrier medium was switched for a 5 min period to 10 % ASW containing mannitol (to maintain an osmolality equal to that of 100 % ASW); (3) the carrier medium was switched back to 100 % ASW and the olfactory response was measured after a 5 min period; (4) the carrier medium was switched to 10 % ASW (without mannitol) for a 5 min period; (5) the carrier medium was returned to 100 % ASW and the olfactory response was tested after 5 min. In some preparations, an additional cycle was added at the end of the above sequence. In these cases, the aesthetascs were exposed to a carrier stream of distilled water for 5 min, then returned to 100 % ASW for retesting the olfactory response.

Flux measurements

The ionic equilibration and continuity between the hemolymph and the sensillar lymph was explored by studying the unidirectional fluxes of [^{14}C]thiocyanate (an extracellular fluid volume marker) and ^{22}Na (the radioactive isotope of a major circulating electrolyte). The experimental approach used in these studies is depicted schematically in Fig. 2. Crabs were secured to a platform and one or both antennules inserted into separate reservoirs, each containing a known volume of water (either sea water or well water, depending on the acclimation status of the animal), which was continuously stirred. A small hole drilled through the carapace just to the right of the heart, and sealed with a piece of latex rubber glued to the carapace with cyanoacrylate, provided access to the pericardial sinus for tracer injection and hemolymph withdrawal. Samples of reservoir water (efflux studies) and hemolymph (efflux and influx studies) were taken periodically during the course of an experiment and the radioactivity was measured by means of liquid scintillation.

The protocol for the efflux experiments was as follows. A 100–500 µl volume of thermoequilibrated tracer [either ^{22}NaCl (0.59–0.89 MBq) or [^{14}C]thiocyanate (0.56–1.48 MBq)] prepared in SW-saline was injected into the pericardial sinus. Hemolymph samples (450 µl) were withdrawn from the same location at 30 or 60 min intervals, and 250 µl of the sample

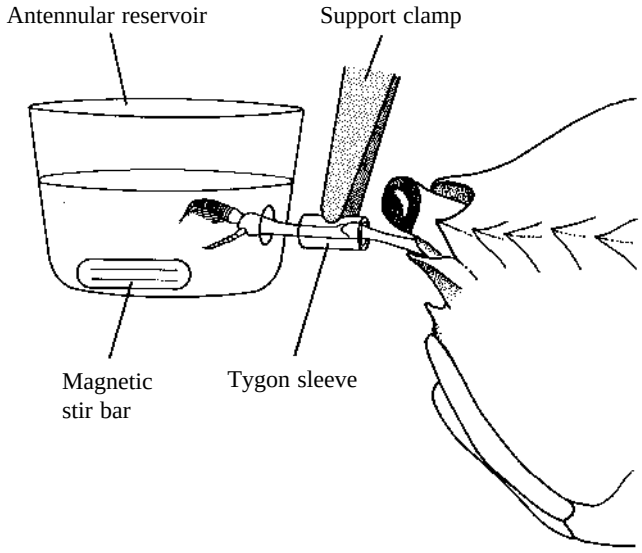

Fig. 2. Schematic diagram of the apparatus used to examine tracer flux between the hemolymph and the sensillar lymph, and to measure trans-antennule potential. The crab (shown to the right) was secured to a stable platform and remained out of water during the course of an experiment.

were expelled into 5 ml of EcoLume fluor for the measurement of radioactivity. Commencing at 60 min following injection of the isotope, 200 µl water samples were taken from each antennular reservoir at 30 min intervals and radioactivity was counted in 5 ml of EcoLume fluor. For influx measurements (Na^+ only), the procedures were similar with the exception that the isotope (0.74 MBq) was introduced to the reservoir and its appearance in the hemolymph was monitored over time (i.e. at 30 min intervals commencing at 60 min following introduction of the isotope to the antennular reservoir).

The aesthetasc-specific component of all flux measurements was based on control preparations in which effluxes from the right and left antennules within the same animal were compared. The tuft of aesthetascs on one of the antennules was coated with either cyanoacrylate or 5 min epoxy, whereas the opposite antennule remained intact. The portion of efflux attributable to the aesthetascs was determined by the difference between the two efflux rates, and the mean values were then used to correct flux measurements made on intact antennules. These determinations yielded the following correction factors (i.e. the portion of the total flux due to the aesthetascs): thiocyanate, SW-acclimated crabs 0.55 (N=5); Na^+, SW-acclimated crabs 0.66 (N=4); Na^+, FW-acclimated crabs 0.36 (N=5).

Trans-antennule potential

The electrical potential across the antennule was measured using a procedure similar to that employed by Wheatly and Henry (1987) for transepithelial potential measurements. Utilizing the apparatus depicted in Fig. 2 (but without the stirring bar), the electrical potential was measured between the hemolymph and the water in the antennular reservoir. A pair of agar bridges (polyethylene tubes filled with 2 % agar in ASW), one inserted into the water of the reservoir and the other into the pericardial sinus of the crab (*via* a small hole drilled in the carapace), were connected to beakers filled with 3 mol l^{-1} KCl and containing matched calomel electrodes.

Whole-animal potentials were also measured in some crabs. For these determinations, the restrained animal was immersed in water (of the acclimation salinity) to a level just below the insertion point of the electrode in the pericardial sinus. The agar bridge of the reference electrode was inserted into the water bathing the crab.

Voltage recordings were made using a Copenhagen Radiometer PHM84 ($10^{12}\,\Omega$ input impedance); all measurements were corrected for bridge and tip asymmetry.

Morphological measurements

To determine how the length of the ODSs was affected by acclimation to brackish water, SW-acclimated crabs were directly transferred to and held for 14 days in a tank with a flow-through supply of 13 % SW. The water supply was provided by a fail-safe system that proportionally diluted ambient sea water with dechlorinated tap water. Measurements of the length of the exposed ODSs (i.e. portions extending beyond the constricted region) were made on five aesthetascs from each animal after 14 days using differential interference contrast (DIC) light microscopy as described previously (Gleeson *et al.* 1996). To gain insight on the time course of length changes in the ODSs, at 7–8 days post-transfer to 13 % SW, one antennule from each of seven animals was removed and the length of the ODSs measured in five aesthetascs. The mean length of the ODSs of these animals was then compared with the mean value for five aesthetascs examined on the opposite antennule at day 14. For detailed morphological examination, electron microscopy was used. Tissue preparation for this procedure was as described by Gleeson *et al*. (1996).

Data

All measurements are expressed as mean ± standard error of mean (S.E.M.). Unless otherwise indicated, all statistical procedures were performed as described by Zar (1984).

Results

Neurophysiological measurements

Extracellular, multiunit activity recorded from axon bundles innervating the aesthetascs of SW-acclimated blue crabs shows an increase with odor (TetraMarin) stimulation that is concentration-dependent (Fig. 3). Comparing FW- and SW-acclimated crabs, a difference in the magnitude of this olfactory response is clearly evident on the basis of analyses using the standardized dilution of TetraMarin; i.e. the level for FW-acclimated animals is approximately 20 % of that of SW-acclimated animals (Fig. 4).

In experiments investigating how the olfactory response of FW-acclimated crabs changed during acclimation to SW,

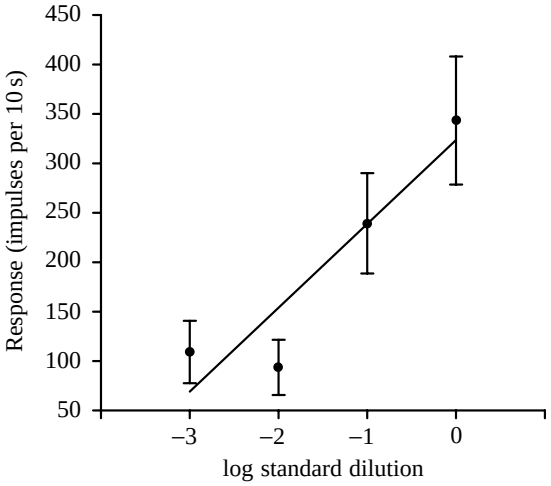

Fig. 3. Olfactory responses (impulses per 10 s) of antennules from seawater (SW)-acclimated crabs as a function of log odor concentration. The odor stimulus was a standard TetraMarin solution diluted as indicated. Linear regression analysis yields r^2=0.3548; the slope is significantly greater than zero (P<0.001). Values are means ± S.E.M. (N=8).

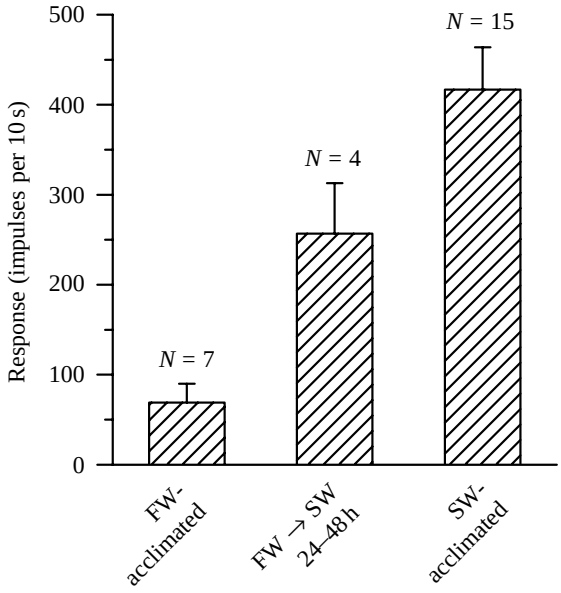

Fig. 4. Olfactory responses of antennules of freshwater (FW)-acclimated and SW-acclimated crabs to the standard TetraMarin odor solution. The middle bar depicts the responses of antennules from FW-acclimated crabs transferred to SW and held for 24–48 h prior to testing [data are the same as those in Fig. 5 (24–48 h group, highest TetraMarin concentration)]. The antennules of the FW-acclimated animals were perfused with FW-saline, and well water was used as the carrier medium in the olfactometer. For the other two groups, the antennules were perfused with SW-saline and artificial sea water (ASW) was used as the carrier medium. A Kruskal–Wallis test coupled with multiple comparisons according to Dunn (Zar, 1984) showed a significant difference between the response of FW-acclimated crabs and those of the other two groups (P<0.05). Values are means + S.E.M.

animals acclimated to FW were transferred directly to SW-tanks and held for varying periods prior to testing the antennules. Within 24–48 h following transfer to SW, there was a significant increase in the response to the TetraMarin standard odor solution (Figs 4, 5). This elevated response was characterized by an increase in the slope of the concentration–response function with time (Fig. 5).

It is important to note that the measurements for the FW-acclimated animals presented in Fig. 4 were made using a perfusion saline and carrier medium that matched the acclimation conditions of the animals. That is, the antennules were perfused with FW-saline and well water was used as the carrier medium in the olfactometer. Antennules of the SW-acclimated crabs, in contrast, were perfused with the SW-saline and ASW was the carrier medium. It might be argued, therefore, that the difference in the olfactory responses between FW- and SW-acclimated animals is simply due to differences in the carrier and/or perfusion media. Arguing against this, however, are the results from antennules of FW-acclimated animals that were perfused with SW-saline and tested using ASW as the carrier medium (filled circles, Fig. 5). The mean response to the highest odor concentration in these trials (the TetraMarin standard concentration) agrees closely with the response (to the same concentration) of antennules from FW-acclimated crabs perfused with FW-saline and tested using well water as the carrier medium (Fig. 4); i.e. 83 and 69 impulses per 10 s, respectively.

Previous work (Gleeson *et al.* 1994) indicated that the aesthetascs of SW-acclimated blue crabs exhibit a significant reduction in olfactory response following an acute 5 min exposure to reduced salinity (50 % and 25 % ASW). To explore the nature of this reduction further, two conditions were examined using the antennules of SW-acclimated crabs. In these studies, the olfactory response was measured in 100 % ASW before and after a 5 min exposure to (1) 10 % ASW (hypo-osmotic, hypo-ionic conditions) and (2) 10 % ASW containing sufficient mannitol to maintain an osmolality equal to that of 100 % ASW (iso-osmotic, hypo-ionic conditions). Exposure of the aesthetascs to 10 % ASW alone resulted in a complete loss of response in six of nine antennule preparations (e.g. Fig. 6A). In the remaining three preparations, only a partial decrease in response was evident (e.g. Fig. 6B); a complete loss of the response occurred, however, after a 5 min exposure to distilled water (Fig. 6B). For all animals tested, the presence of mannitol in the 10 % ASW prevented a decrease in the olfactory response (Fig. 6A,B). The complete loss of response in 10 % ASW *versus* 10 % ASW containing mannitol is significant (Fisher exact test P=0.01).

Morphological studies complementing these physiological experiments demonstrated that the exposed ODSs are osmotically ablated when the aesthetascs of SW-acclimated crabs are immersed in 10 % ASW for 5 min. Following such treatment, the interior of the aesthetasc, beyond the constricted region, takes on a granular appearance when viewed with DIC optical microscopy. An examination using transmission

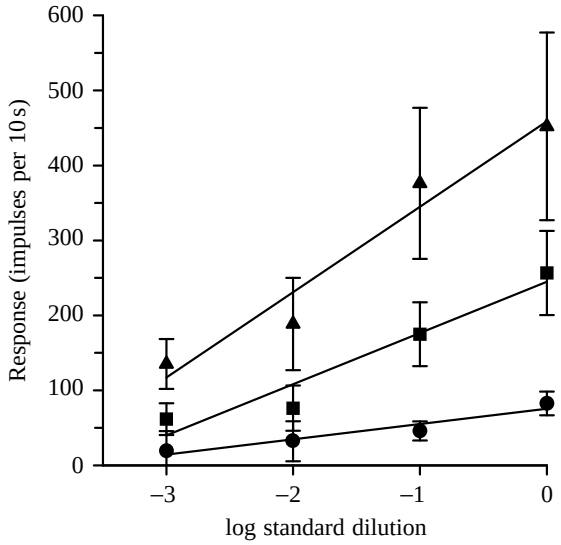

Fig. 5. Olfactory responses of crab antennules as a function of odor concentration. The odor stimulus was a standard TeraMarin solution diluted as indicated. All antennules in this study were perfused with SW-saline and ASW was used as the carrier medium in the olfactometer. ● FW-acclimated crabs (N=3); ■ FW-acclimated crabs transferred to SW and held for 24–48 h (N=4); ▲ FW-acclimated crabs transferred to SW and held for 96 h (N=3). Linear regression analyses yield r^2 values of 0.3522, 0.5307 and 0.5008, respectively; all slopes are significantly greater than zero (P<0.05). The slopes of the two concentration–response functions for FW-acclimated crabs transferred and held in SW are both significantly greater than that for FW-acclimated animals [test for parallelism: P<0.05 (Pharmacologic Calculation System, Life Science Associates)]. Values are means ± S.E.M.

electron microscopy reveals extensive disruption of the ODSs after a 15 min exposure to 10 % ASW; this is characterized by the formation of small membrane vesicles (Fig. 7). If mannitol is present in the 10 % ASW to maintain conditions iso-osmotic to 100 % ASW, the structural integrity of the ODSs is clearly preserved. This is indicated by retention of the characteristic longitudinal profiles of the ODSs as viewed using DIC microscopy (Gleeson *et al.* 1996).

Thiocyanate flux

Continuity between the hemolymph and the sensillar lymph bathing the ODSs of aesthetascs was explored using thiocyanate, a commonly used extracellular fluid marker (e.g. Gleeson and Zubkoff, 1977). [^{14}C]thiocyanate injected into the pericardial sinus of SW-acclimated, male blue crabs rapidly distributed within the extracellular fluid (=hemolymph) compartment, reaching equilibrium by 60 min post-injection, and remained at a relatively constant level over the 2 h period of the experiment (inset, Fig. 8). Samples of water taken at 30 min intervals (commencing at 60 min post-injection) from the antennular reservoir showed a linear increase in radioactivity (=thiocyanate) with time (Fig. 8). A regression analysis was used to determine the rate of this ‘leakage’ or efflux from the antennule, which was in turn corrected to yield the aesthetasc-specific efflux rate (see Materials and methods).

If this efflux of [^{14}C]thiocyanate is viewed as a percolation of extracellular fluid from the hemolymph through the aesthetascs, with no change in thiocyanate concentration, then an estimate of the fluid volume (possibly representing sensillar lymph) generated within each aesthetasc per unit time can be

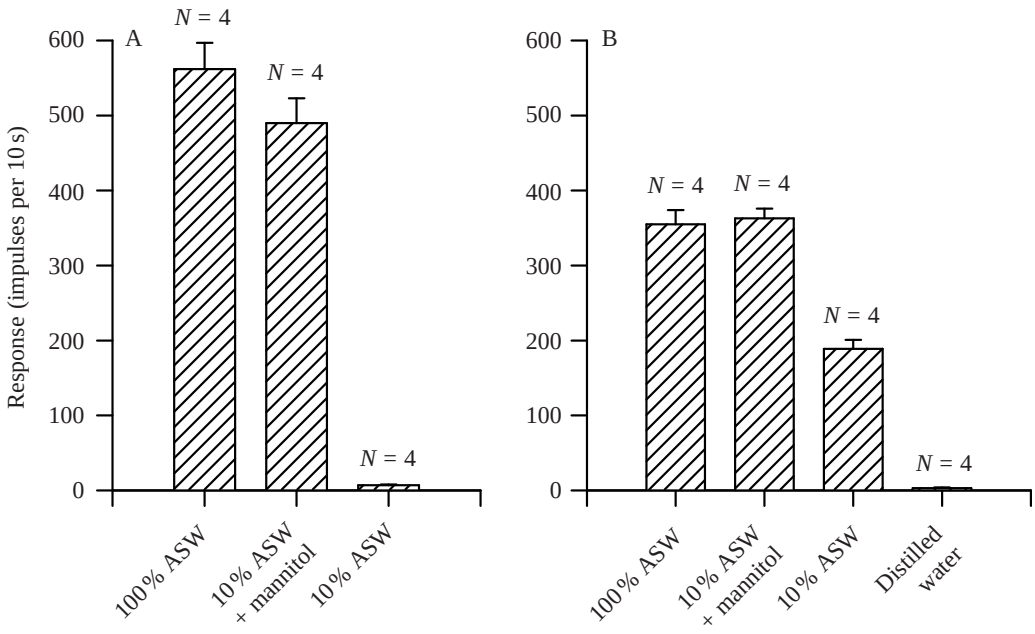

Fig. 6. (A,B) Olfactory responses of antennules from SW-acclimated crabs following acute exposure to low salinity in the presence and absence of iso-osmotic conditions. Data are from two representative antennule preparations. The response to the odor stimulus (TetraMarin standard) was measured in 100 % ASW (replicated four times) following a 5 min exposure to each of the indicated solutions (see Materials and methods for protocol details). (A) Antennule preparation in which the exposure to 10 % ASW containing 0.82 mol l^{-1} mannitol (to maintain osmotic conditions equal to those of 100 % ASW) had little effect on the olfactory response. Exposure to 10 % ASW without added mannitol, however, resulted in a complete loss of response. (B) Antennule preparation in which the olfactory response was unaffected by exposure to 10 % ASW containing 0.82 mol l^{-1} mannitol. Exposure to 10 % ASW without added mannitol partially reduced the response; distilled water eliminated it. Values are means + S.E.M.

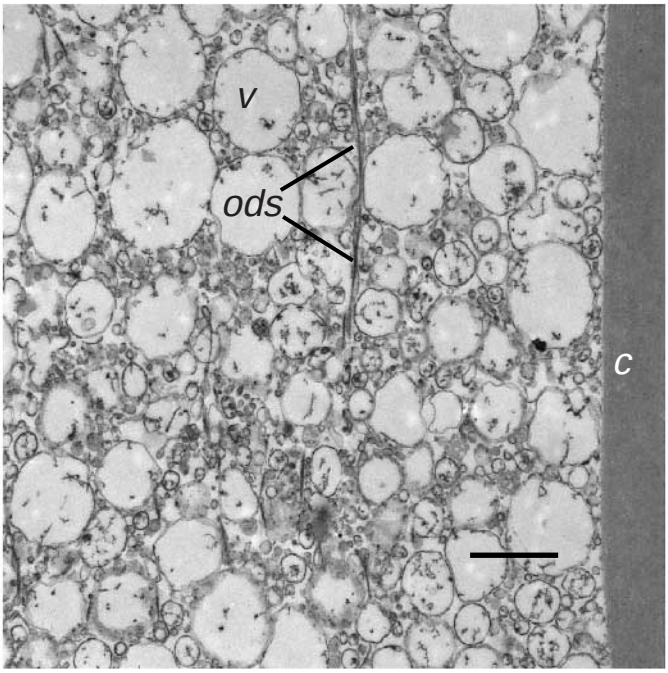

Fig. 7. Transmission electron micrograph of an aesthetasc (longitudinal section) from a SW-acclimated blue crab. The antennule was exposed to 10 % ASW for 15 min followed by a return to 100 % ASW immediately prior to fixation. *c*, cuticle; *v*, membrane vesicle; *ods*, fragment of outer dendritic segment. Scale bar, 1 μm.

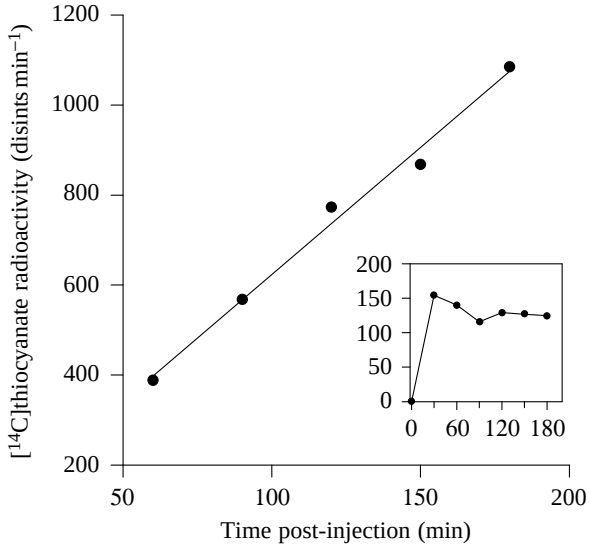

Fig. 8. Example of [^{14}C]thiocyanate efflux from the antennule of a SW-acclimated blue crab. The radioactivity present in the water of the antennular reservoir is shown as a function of time following injection of [^{14}C]thiocyanate into the hemolymph. The inset shows the level of [^{14}C]thiocyanate radioactivity ($10^3\times$ disints min^{-1} 250 μl^{-1}) in the hemolymph as a function of time (min) post-injection.

made. This value was computed by dividing the mean level of radioactivity per unit volume of hemolymph at equilibrium into the aesthetasc-specific radioactivity 'leaked' from the antennule per unit time, then dividing by 700 (the number of aesthetascs on each antennule; Gleeson, 1982). On the basis of data obtained from eight animals, the mean rate of fluid generation was calculated to be 115±11 fl s^{-1} aesthetasc^{-1}.

Na$^+$ flux

Ion exchange between the hemolymph and sensillar lymph was investigated by studying the unidirectional fluxes (efflux and influx) of Na^+, a major circulating electrolyte. The experimental approach was similar to that employed for thiocyanate. As with thiocyanate, ^{22}Na injected into the pericardial sinus of experimental crabs was rapidly distributed within the hemolymph, reaching a plateau by 60 min post-injection, and remained at a relatively constant level for the remaining 2 h of the experiment (inset, Fig. 9). Measurements of radioactivity in the reservoir containing the antennule showed a linear increase with time (Fig. 9). Using this procedure, and based on the specific activity of Na^+ in the hemolymph, the aesthetasc-specific Na^+ effluxes for SW-acclimated and FW-acclimated crabs were determined. There was a significantly lower level of Na^+ efflux in FW-acclimated animals (Fig. 10).

To assess the net movement of Na^+ through the aesthetascs of FW-acclimated crabs, the aesthetasc-specific influx of ^{22}Na from the antennular reservoir to the hemolymph was measured; these studies generated a value of 1.68±0.27 fmol s^{-1} aesthetasc^{-1} (N=5). Subtracting influx from efflux (43.85±6.5 fmol s^{-1} aesthetasc^{-1}, N=6) yields a net efflux of Na^+ from the aesthetascs of FW-acclimated animals of 42.17 fmol s^{-1} aesthetasc^{-1}. For the purposes of comparison with thiocyanate flux, if this net outward movement of Na^+ is viewed as a percolation of extracellular fluid with a Na^+ concentration equivalent to that of hemolymph (i.e. 429 mmol l^{-1}), the volume of fluid generated per unit time would be 98 fl s^{-1} aesthetasc^{-1}.

Trans-antennule potential

To investigate the nature of Na^+ movement through the aesthetascs, both trans-antennule and whole-animal electrical potentials were measured in SW- and FW-acclimated blue crabs. For SW-acclimated animals, the potential of the hemolymph relative to the sea water of the antennular reservoir was −1.66±0.17 mV (N=6); whole-animal measurements (i.e. the potential between the hemolymph and the sea water bathing the entire crab) yielded a value of −0.75±0.13 mV (N=6). In FW-acclimated crabs, these measurements were −59±3.8 mV (N=8) and −25±1.4 mV (N=5), respectively. In the latter group, well water served as the bathing medium in the antennular reservoir and as the medium in which whole crabs were immersed.

Length of the ODSs in brackish water

To explore how acclimation to brackish water affects the length of the exposed ODSs in aesthetascs, SW-acclimated animals were transferred directly to tanks with a flow-through

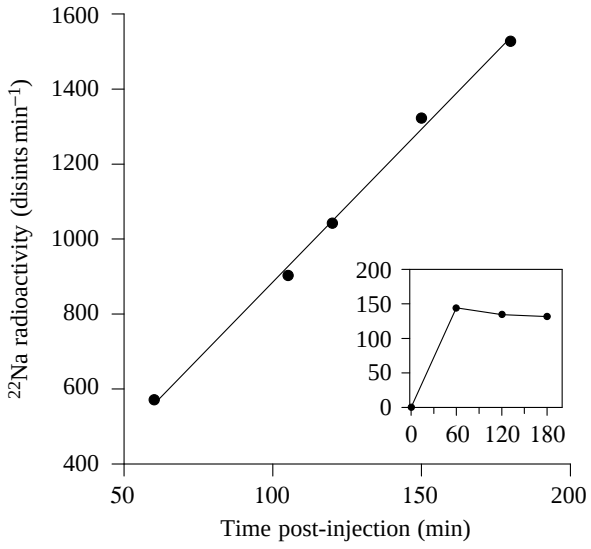

Fig. 9. ^{22}Na efflux from the antennule of a FW-acclimated blue crab. The radioactivity present in the water of the antennular reservoir is shown as a function of time from injection of ^{22}NaCl into the hemolymph. The inset shows the level of ^{22}NaCl radioactivity ($10^3\times$ disints min^{-1} 250 µl^{-1}) in the hemolymph as a function of time (min) post-injection.

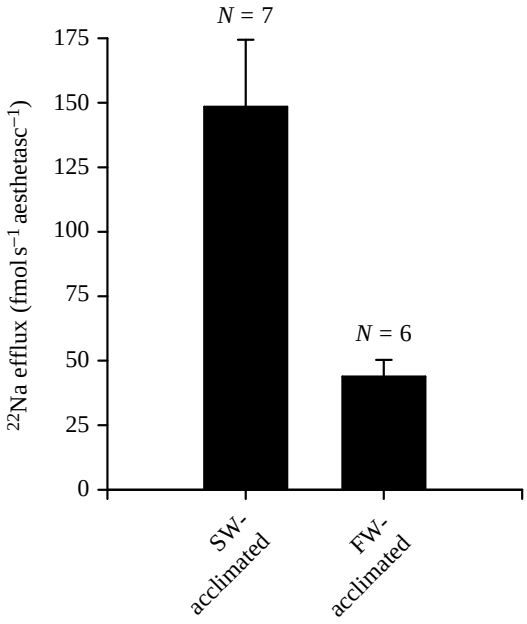

Fig. 10. Aesthetasc-specific efflux of ^{22}Na from SW- and FW-acclimated blue crabs. Determinations based on measurements of ^{22}Na efflux from the hemolymph *via* the aesthetascs and knowing the specific activity of ^{22}Na in the hemolymph (specific activity based on Na^+ concentrations of 468 mmol l^{-1} and 429 mmol l^{-1} in the hemolymph of SW-acclimated and FW-acclimated crabs, respectively). All efflux determinations on SW-acclimated crabs were made using sea water in the antennule-containing reservoir; for FW-acclimated crabs, the reservoir contained well water. The mean values are significantly different (*t*-test, $P<0.01$). Values are means + S.E.M.

supply of 13% SW (salinity 4.7‰) and held in these conditions for 14 days. Measurements using DIC microscopy at the end of this period revealed ODS lengths intermediate between those of FW-acclimated and SW-acclimated animals (Fig. 11). The adjustment in length apparently occurred within 1 week following transfer to 13% SW. This was supported by the observation that, within individual animals, there was no significant change in ODS length for aesthetascs inspected at 7–8 days post-transfer to 13% SW *versus* those examined at 14 days post-transfer (284±26 µm *versus* 279±16 µm, respectively; paired *t*-test, $P=0.43$, $N=7$).

Discussion

The results of the present study, together with the morphological findings reported previously (Gleeson *et al.* 1996), provide insight into the challenges of maintaining olfactory function at low salinities and how these challenges might be met in the aesthetascs of the blue crab. First, the neurophysiological results show that the olfactory response parallels morphological changes occurring in the ODSs as the crab acclimates to (or is acutely subjected to) different salinities. Second, the measurements of association between the hemolymph and sensillar lymph provide the basis for a hypothesis on how olfaction might be supported in challenging ionic/osmotic conditions.

Our physiological results suggest that the olfactory response, which is significantly reduced in blue crabs living in fresh water, is closely linked to the level of ODS exposure to the odor environment. Three observations support this idea. (1) Using a standard odor stimulus, a comparison of the olfactory response between FW-acclimated and SW-acclimated crabs reveals a substantial difference in magnitude. This difference is reflected by a comparable difference in ODS length (compare Fig. 4 and Fig. 11). (2) Following transfer of FW-acclimated animals to SW, there is a significant increase in the olfactory response at 24–48 h (Fig. 4). Here too there is a morphological correlate; namely, at 48 h post-transfer of FW-acclimated crabs to SW, the length of the ODSs has increased significantly [mean lengths: FW-acclimated, 150±8 µm ($N=41$); after 48 h in SW, 300±13 µm ($N=23$) (from Gleeson *et al.* 1996)]. (3) The increase in the olfactory response with acclimation to SW is characterized by an increase in the slope of the concentration–response function (Fig. 5). By 96 h following transfer of FW-acclimated crabs to SW, the slope is similar to that for SW-acclimated animals (compare the slopes of Fig. 3 and the top line in Fig. 5). Again, this physiological variable roughly corresponds with ODS length; i.e. by 96 h following transfer of FW-acclimated crabs to SW, the length of the ODSs approaches that of SW-acclimated animals (Gleeson *et al.* 1996).

Considered together, these observations suggest that the magnitude of the olfactory response is linked to the length of the exposed ODSs. It is likely that longer ODS length, together with associated branching of the ODSs, increases the

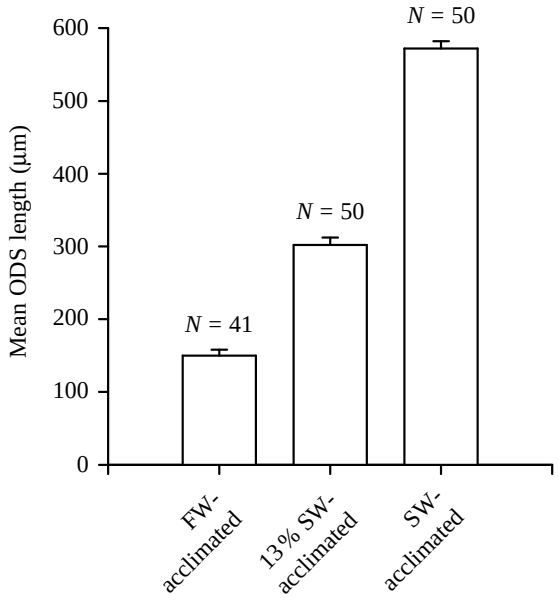

Fig. 11. Length of the exposed outer dendritic segments (ODSs) in blue crabs acclimated to 13 % SW compared with FW- and SW-acclimated animals. Data for FW-acclimated animals are taken from Gleeson *et al.* (1996). All means are significantly different (one-way ANOVA with Tukey's *post-hoc* test, $P<0.001$). Values are means + S.E.M.

membrane surface area presented to the odor environment, thereby increasing the number of odor receptors and ion channels contributing to the graded receptor potential. Further study will be required to determine whether the reduced olfactory response (sensitivity) at low salinities translates to a corresponding reduction in the behavioral responses to odors. It is conceivable that compensatory mechanisms within the central nervous system are able at least partially to offset decreases in peripheral sensitivity.

The ODSs of SW-acclimated crabs are vulnerable to the osmotic effects of rapid reductions in salinity. Previous work showed that 5 min exposures to reduced salinity (50 % and 25 % ASW) substantially decreased the olfactory response subsequently tested in 100 % ASW (Gleeson *et al.* 1994). In the present study, we found that short-term exposure to 10 % ASW resulted in partial or complete loss of the response in a majority of the animals tested, and this was correlated with morphological evidence of osmotic ablation. The presence of mannitol in the 10 % ASW (to maintain an osmolality equal to that of 100 % ASW), preserved the olfactory response as well as the structural integrity of the ODSs. These findings clearly show that the ODSs of SW-acclimated crabs are highly vulnerable to changes in environmental osmotic conditions. This is not surprising considering the anatomical data and results from dye studies indicating uniform permeability of the aesthetasc cuticle beyond the constricted region (Gleeson *et al.* 1996). The ODSs are apparently less vulnerable to changes in ionic conditions.

The results of the experiments examining radiotracer movements across the aesthetascs not only suggest that there is continuity between the hemolymph and sensillar lymph but also provide a quantitative estimate of the fluxes. The findings suggest a mechanism by which olfactory function might be sustained at low salinities. In these experiments, both [^{14}C]thiocyanate and ^{22}Na 'leaked' from the hemolymph to the water of the antennular reservoir. Assuming that the aesthetasc-specific component of these measurements represents movement from the hemolymph through the aesthetascs by way of the sensillar lymph, an estimate of the efflux per aesthetasc can be made. Of particular interest is the net outward movement of Na^+ in FW-acclimated crabs; this was determined to be 42.17 fmol s^{-1} aesthetasc^{-1}. We propose that this steady, net efflux of Na^+ (and probably of other ions as well) generates an ionic/osmotic microenvironment within the aesthetasc that is critically important for maintaining the structural and functional integrity of the exposed ODSs in low-salinity conditions (Fig. 12). To help place this flux into perspective, if the sensillar lymph bathing the exposed ODSs in FW-acclimated crabs is assumed to have a Na^+ concentration comparable to that of hemolymph (429 mmol l^{-1}), and given the volume of sensillar lymph occupied by the ODSs (about 12 pl), this rate of Na^+ efflux would be sufficient to replace the total Na^+ in this volume (5.1 pmol) approximately every 2 min.

To clarify the specific mechanism of Na^+ efflux from aesthetascs will require further study; however, some insight can be gained from a consideration of the electrical potential across the antennules. Two lines of evidence suggest that the Na^+ efflux in fresh water may be driven largely by diffusion down the concentration gradient established between the hemolymph and the external medium – a gradient maintained by active transport of NaCl into the hemolymph *via* specialized epithelia of the gills (Cameron, 1978; Neufeld *et al.* 1980; Gilles and Pequeux, 1986; Pequeux *et al.* 1988; Burnett and Towle, 1990). First, the observed flux ratio for Na^+ (i.e. influx/efflux=1.68/43.85=0.038) falls within the range of that predicted for passive diffusion based on the Ussing (flux ratio) equation:

$$\ln(J_i/J_o) = \ln(C_o/C_i) - zF/\boldsymbol{R}T \times V, \quad (1)$$

where J_i and J_o are the influx and efflux rates, respectively, C_o and C_i are the concentrations of Na^+ in the external medium and hemolymph, respectively, z is the valence of Na^+, F is the Faraday constant, $\boldsymbol{R}$ is the gas constant, T is the absolute temperature and V is the electrical potential across the antennule. The predicted flux ratio at 25 °C (using C_o=4.4 mmol l^{-1} and C_i=429 mmol l^{-1}) is 0.101 for the trans-antennule electrical potential of −59 mV. This calculated value is within the same order of magnitude as the observed ratio (0.038), suggesting that the outward movement of Na^+ from aesthetascs is a passive process. The somewhat lower observed value may reflect an additional active transport component, but could also derive entirely from flux coupling such as that occurring in ion diffusion through long pores (Hodgkin and Keynes, 1955; Heckmann, 1972; Hille and Schwarz, 1978).

A second relationship, which is consistent with passive movement of Na^+ through the aesthetascs, emerges from a comparison of the unidirectional effluxes for SW- and FW-

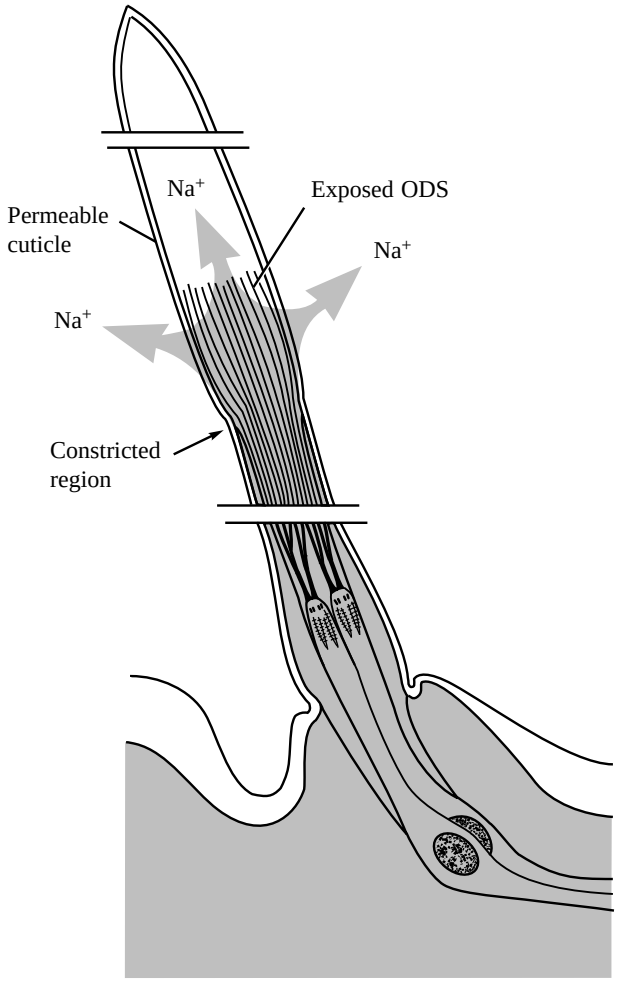

Fig. 12. Schematic diagram of a single aesthetasc sensillum (from a FW-acclimated crab) illustrating the proposed pathway of net Na^+ flux from the hemolymph to the sensillar lymph and ultimately its diffusive loss across the permeable cuticle of the aesthetasc. The exposed ODSs extending beyond the constricted region are bathed in a fluid microenvironment (sensillar lymph) in which the Na^+ concentration is maintained by a diffusion-generated flux from the hemolymph that matches the diffusive loss to the external environment.

acclimated crabs. Namely, the reduced efflux measured in FW-acclimated animals approximates the value predicted from the efflux rates of SW-acclimated animals, assuming simple diffusion down a potential gradient. If the value for aesthetasc-specific efflux in SW-acclimated crabs is proportionally adjusted to account for the Na^+ concentration difference between the hemolymph of FW- and SW-acclimated crabs (i.e. 429/468=0.92), this yields an efflux of 136.57 fmol s^{-1} aesthetasc^{-1}. The effect of a potential on this flux can be calculated using the following relationship, assuming a simple diffusion model:

$$r_o = P_{Na} z V' / (1 - e^{-zV'}), \quad (2)$$

where r_o is the rate constant for outward Na^+ movement, P_{Na} is the permeability constant for Na^+, and $V'=VF/\boldsymbol{R}T$ (Kuffler *et al.* 1984). This yields a factor by which the rate constant, and therefore flux, is affected by a given potential; at 25 °C, for a change in trans-antennule potential from −1.66 mV to −59 mV, this factor is 0.254. Therefore, the predicted aesthetasc-specific efflux value for FW-acclimated crabs would be 34.74 fmol s^{-1} aesthetasc^{-1}. Note that this *predicted* flux value, based on efflux for SW-acclimated crabs, approximates the *measured* level of 43.85 fmol s^{-1} aesthetasc^{-1} in FW-acclimated animals.

To determine the exact pathway(s) by which Na^+ moves from the hemolymph to the sensillar lymph will require additional experimentation. The observation that thiocyanate traverses the aesthetascs argues for extracellular continuity between these two fluid compartments; however, facilitated diffusion and/or active transport processes across the sensory epithelium cannot be ruled out and may account for the differences between the calculated and observed flux levels discussed above. Further study will be necessary to resolve these questions. For example, experiments at the ultrastructural level using electron-dense extracellular markers would provide insight into the nature of the apparent continuity between the hemolymph and sensillar lymph.

The rate of Na^+ efflux through the aesthetascs of crabs living in fresh water is sufficient to renew the Na^+ present in the sensillar lymph continuously and rapidly, yet the total amount of Na^+ lost by this efflux over a 24 h period is only a small fraction of that available in the hemolymph. Assuming that the voltage across the antennules primarily represents a diffusion potential, it may largely derive from a differential permeability to Cl^- (relative to Na^+), as has been suggested for gill potentials in fish (Eddy, 1975; Potts, 1984). The magnitude of the potential and its implications for Na^+ flux have been discussed with reference to potentials measured across the isolated antennule. Although useful for interpreting these experimental flux relationships, in order to estimate the absolute levels of antennular flux in free-living animals, the potential existing across the whole animal is the relevant measure. Whole-animal, transepithelial potentials for blue crabs acclimated to low salinity (2‰) or to fresh water are reported to be approximately −3 mV (Cameron, 1978; Neufeld and Cameron, 1992). Interestingly, our measurements yielded a substantially larger potential difference (i.e. −25 mV), which may derive in part from the lower Ca^{2+} level of the well water (Cameron, 1978). For a given potential, the rate-constant equation presented above (equation 2) can be used to estimate the aesthetasc-specific outward movement of Na^+ in free-living crabs; the relatively low level of influx can be ignored in this calculation. An upper estimate of the efflux (i.e. based on the measured level of 43.85 fmol s^{-1} aesthetasc^{-1} and considering a change in potential from −59 mV to −3 mV) gives 167.77 fmol s^{-1} aesthetasc^{-1}. This flux rate would be sufficient to replace the Na^+ of the sensillar lymph occupied by the exposed ODSs approximately every 30 s (compared with the 2 min rate of turnover calculated above). Such a rate of Na^+ loss from both antennules over a 24 h period in a 140 g crab (assuming a hemolymph volume of 35 ml and a Na^+

concentration of 429 mmol l^{-1}) represents only 0.1 % of the total Na^+ present in the hemolymph.

We postulate that the reduced length of the ODSs in FW-acclimated crabs directly reflects the limited region in which an extracellular microenvironment suitable for maintaining neural function can be sustained within the aesthetasc. In essence, it reflects the volume of sensillar lymph in which an equilibrium between diffusive loss of ions to the external milieu and replacement by efflux from the hemolymph produces ionic/osmotic conditions compatible with sustaining the structural and functional integrity of the exposed ODSs (Fig. 12). Indeed, this microenvironment may very well approximate the ionic/osmotic conditions of the hemolymph. The importance of continuously maintaining this environment within the aesthetascs is indicated by the rapid breakdown of the exposed ODSs in the event that the link to the hemolymph is compromised. If, for example, the aesthetascs of FW-acclimated crabs are dissected from the antennule in fresh water (the acclimation medium), the ODSs immediately vesiculate (Gleeson *et al.* 1996). These observations suggest that the ODSs of FW-acclimated crabs, like those of SW-acclimated animals, are vulnerable to conditions of low osmotic concentration, but are 'protected' by a fluid milieu which is maintained dynamically. We propose that the length of the exposed ODSs at low salinities is directly related to the distance over which this microenvironment can be supported; thus, as salinity increases, this distance extends further distally, thereby sustaining longer portions of exposed ODSs. Such a model is certainly consistent with the finding that the ODSs of crabs acclimated to brackish waters are significantly longer than those of FW-acclimated animals (Fig. 11).

In summary, our findings provide insight into what is perhaps the major challenge to maintaining olfaction at low salinities; namely, facilitating the access of odors to chemosensory dendrites while simultaneously sustaining neural function in those dendrites. The results shed light on how this problem might be solved in aquatic organisms. We propose that the blue crab meets the challenge of low salinity by dynamically generating an ionic/osmotic microenvironment within the aesthetascs – a microenvironment that is sustained by a continuous efflux of Na^+ (and other ions) from the hemolymph. The exact mechanism by which the Na^+ transfer is mediated remains to be determined; however, our findings suggest that passive diffusion down a concentration gradient established between the hemolymph and external medium ultimately drives the net outward flux. We propose that, at low salinities, the length of the exposed ODSs, and consequently olfactory sensitivity, is constrained by the maximum distance over which this diffusion-generated microenvironment can be effectively maintained.

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