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Adaptation to reduced salinity affects the olfactory sensitivity of Senegalese sole (*Solea senegalensis* Kaup 1858) to Ca^{2+} and Na^{+} but not amino acids

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SUMMARY

The Senegalese sole is a marine flatfish, which often penetrates into estuarine waters to feed. It cannot, however, survive in full freshwater. The current study investigated the effect of adaptation to low salinity (10‰) on olfactory responses to changes in environmental $[Ca^{2+}]$ and $[Na^{+}]$ and amino acids by the electro-encephalogram (EEG) recorded from the olfactory bulb. The sole showed olfactory responses to increases in environmental $[Na^{+}]$ and decreases in environmental $[Ca^{2+}]$; sensitivity to Na^{+} was greater at 10‰ whereas sensitivity to Ca^{2+} was greater at 35‰. Decreased environmental $[Na^{+}]$ increased sensitivity to changes in $[Ca^{2+}]$ whereas increased environmental $[Ca^{2+}]$ decreased bulbar responses to changes in $[Na^{+}]$. Sensitivity to amino acids was unaffected by external salinity. However, the absence of external Na^{+} strongly decreased bulbar responses to amino acids in fish adapted to 35‰ seawater but not in those at 10‰. The absence of external Ca^{2+} had no such effect at either salinity. This suggests that odorant-receptor binding and/or olfactory transduction is reliant on external Na^{+} (but not Ca^{2+}) at higher salinities but the olfactory system is able to adapt to lower environmental $[Na^{+}]$. Taken together, these results suggest that reductions of external salinity modulate olfactory sensitivity to environmental Ca^{2+} and Na^{+} but not amino acids. However, at low salinities, olfactory sensitivity to amino acids is maintained by decreasing reliance on external Na^{+}.

Key words: olfaction, calcium, sodium, salinity, amino acid, flatfish.

INTRODUCTION

Maintenance of constant plasma $[Ca^{2+}]$ and $[Na^{+}]$ is crucial to vertebrates; both ions are involved in a multitude of physiological processes (e.g. muscular contraction, action potential generation and cellular signalling). In the aquatic environment, levels of these ions can vary from vastly in excess of physiological needs (e.g. seawater) to nearly zero (soft freshwater). As yet, however, the mechanisms by which fish perceive these environmental levels are poorly understood. Accumulating evidence suggests that teleosts have olfactory sensitivity to changes in environmental $[Ca^{2+}]$ and $[Na^{+}]$ (Bodznick, 1978; Hubbard et al., 2000; Hubbard et al., 2002; Nearing et al., 2002). Olfactory sensitivity to changes in environmental $[Ca^{2+}]$ has been described in both freshwater and marine fish; goldfish (*Carassius auratus*) and freshwater-reared sockeye salmon (*Oncorhynchus nerka*) have olfactory sensitivity to increases in environmental $[Ca^{2+}]$ (Bodznick, 1978; Hubbard et al., 2002) whereas the seabream (*Sparus aurata*) is sensitive to reductions in $[Ca^{2+}]$ (Hubbard et al., 2000). Bodznick proposed that the olfactory sensitivity of freshwater-reared sockeye salmon to changes in environmental $[Ca^{2+}]$ contributed to their ability to identify natal rivers (Bodznick, 1978). However, goldfish and seabream do not undergo diadromous migrations, which suggests that there may be other reasons for fish to be able to monitor environmental $[Ca^{2+}]$ (Hubbard et al., 2000; Hubbard et al., 2002). Thus, seabream, although predominantly marine, can survive in brackish water but not in freshwater, and it has been suggested that olfactory sensitivity to changes in environmental $[Ca^{2+}]$ is used to warn the fish that it is reaching the limit of its salinity tolerance (Hubbard et al., 2000; Nearing et al., 2002). It may also affect the physiological mechanisms regulating internal calcium homeostasis.

Goldfish and sockeye salmon also have olfactory sensitivity to increases in environmental $[Na^{+}]$ (Bodznick, 1978; Hubbard and Canário, 2007; Hubbard et al., 2002). The physiological significance of this phenomenon is not yet known; however, the relatively high detection threshold for Na^{+} suggests that this sensitivity is not involved in routine monitoring but may serve as a warning that the fish is approaching the limits of its osmoregulatory capacity and/or activate osmoregulatory mechanisms (Hubbard et al., 2002). Nevertheless, in the goldfish at least, Ca^{2+} and Na^{+} are detected by distinct olfactory mechanisms (Hubbard and Canário, 2007).

As well as acting as putative odorants in their own right, both Ca^{2+} and Na^{+} are intimately involved in olfactory transduction of 'conventional' odorants (Menini, 1999; Nakamura, 2000; Schild and Restrepo, 1998). How the large changes in external availability of these ions that occur as a result of salinity changes may affect olfactory sensitivity has been little studied. In euryhaline fish, such as salmonids, olfactory sensitivity to amino acids seems to be largely independent of external salinity (Shoji et al., 1994; Shoji et al., 1996). However, in the seabream, short-term exposure to Ca^{2+}-free seawater caused a temporary reduction in the olfactory response to L-serine (Hubbard et al., 2000). Thus, olfactory transduction in fish may be independent of external Ca^{2+} and Na^{+} or they may be able to adapt to changing levels. If and how olfactory sensitivity to these ions changes with external salinity has not yet been investigated.

The Senegalese sole (*Solea senegalensis* Kaup 1858), hereafter 'sole', is a marine flatfish of the Mediterranean Sea and north-east Atlantic Ocean. It often penetrates estuarine waters to feed (Cabral,

2000), although it cannot survive in full freshwater. As it is nocturnal and its main prey live buried in the substrate (Bayarri et al., 2004; Cabral, 2000), it is likely that olfaction plays an important role in food-search. Previous work has shown that the amino acids L-phenylalanine and glycine are released by one of the sole's main prey and that the sole has olfactory sensitivity to both (Velez et al., 2005; Velez et al., 2007b). The current study, therefore, investigated how adaptation to reduced salinity may affect olfactory responses to Ca^{2+}, Na^{+}, L-phenylalanine and glycine and the reliance on external Ca^{2+} and Na^{+} in olfactory transduction.

MATERIALS AND METHODS

Experimental animals

Sole were obtained from local aquaculture facilities (IPIMAR, Olhão, Portugal). Fish were grown according to procedure described by Dinis et al. (Dinis et al., 1999) and fed daily on commercial pellets (AQUASOJA 2–3.5 mm, Sorgal SA, Portugal). Experiments were carried out on fish kept in full seawater (35‰) and fish adapted to low-salinity seawater (10‰). Adaptation to low salinity was carried out gradually over one week; sole were then kept at 10‰ for one week prior to use. At the time of experiments animals were between 100 and 300 g.

Recording the electro-encephalogram (EEG) from the olfactory bulb

Prior to recording, fish were anaesthetised by immersion in water containing 200 mg l^{-1} MS-222 (3-aminobenzoic acid ethyl ester; Sigma-Aldrich, Madrid, Spain) followed by intramuscular injection of gallamine triethiodide (Sigma-Aldrich; 0.6 mg 100 g^{-1} body mass) and placed on a padded surface with a slight forward tilt (to prevent water from entering the wound). The gills were irrigated with a constant flow (~1 ml g^{-1} body mass min^{-1}) of aerated water (of the appropriate salinity) containing MS-222 (100 mg l^{-1}). The body of the fish was covered by damp paper towel and the eyes covered with small pieces of black polythene. The olfactory rosette was exposed by cutting the skin and connective tissue covering the epithelium. The nostril was constantly irrigated with charcoal-filtered seawater (without anaesthetic) under gravity (flow-rate: 6–8 ml min^{-1}) *via* a glass tube. Test solutions were delivered to the tube irrigating the nasal cavity *via* a computer-operated three-way solenoid valve for a period of 5 s. The upper (right) olfactory bulb was exposed by removal of the skin, connective tissue and the overlying bone. The electro-encephalogram (EEG) was recorded with a purpose-built 'suction' electrode (Brierley et al., 2001; Hubbard and Canário, 2007) connected to a Neurolog NL104 AC pre-amplifier (Digitimer Ltd, Welwyn Garden City, UK). The electrode was placed lightly on the surface of the olfactory bulb that gave strong responses to both amino acids and ions; this was usually the anterior–lateral portion of the bulb, close to the olfactory nerve. However, the internal anatomy of the sole meant that access to the whole bulb was restricted; attempts to record good bulbar responses to bile acids, for example, were unsuccessful, even though this fish gives large responses to bile salts when assessed by electro-olfactogram (EOG) recording from the olfactory epithelium (Velez et al., 2007a; Velez et al., 2009). Thus, the poor bulbar responses to bile acids were most probably due to the area of the bulb responding best to bile acids being inaccessible to the recording electrode. The signal was filtered (low-pass 300 Hz, high-pass 3 Hz; Neurolog NL125, Digitimer Ltd) and integrated (time constant 1 s, Neurolog NL703, Digitimer Ltd). Both the direct and integrated signals were digitised (Digidata 1300A, Molecular Devices Corporation, Sunny Vale, CA, USA) and displayed on a computer running Axoscope 9.2 software (Molecular Devices Corporation). All surgical and experimental procedures followed the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series No. 123) and the Guidelines for the Use of Fishes in Research by the American Fisheries Society (http://www.fisheries.org/afs/docs/policy_guidelines2004.pdf).

Stimulus solutions

Artificial seawaters (ASW) at 35‰ and 10‰, with and without calcium, with and without sodium, and with and without choline were prepared with the composition shown in Table 1. Ranges of $[Ca^{2+}]$ (10–0 mmol l^{-1}) or $[Na^{+}]$ (0–460 mmol l^{-1}) at 35‰ or 10‰ were made up by appropriate mixing of these solutions. Choline chloride was used to maintain osmolality and $[Cl^{-}]$ in Ca^{2+}- and Na^{+}-free solutions. Thus, the only difference in ionic composition between ASW and Na^{+}- and/or Ca^{2+}-free ASW is that Ca^{2+} and Na^{+} were replaced by choline. Control experiments using Ca^{2+}- and Na^{+}-free ASW without choline and ASW plus 20 mmol l^{-1} choline chloride as a stimulus were also carried out.

When testing the olfactory sensitivity in the absence of external Ca^{2+}, the background water perfusing the olfactory epithelium was Ca^{2+}-free ASW (10‰ or 35‰ as appropriate). When assessing the olfactory sensitivity in the absence of external Na^{+}, the background water superfusing the olfactory epithelium was Na^{+}-free ASW (10‰ or 35‰ as appropriate). When assessing the effect of different $[Na^{+}]$ on the olfactory response to changes in $[Ca^{2+}]$, the background $[Na^{+}]$ was held constant at a given concentration (ranging from 0 to 460 mmol l^{-1}); the response was recorded to a reduction in $[Ca^{2+}]$ from 10 to 0 mmol l^{-1}. Conversely, when assessing the effect of different $[Ca^{2+}]$ on the response to Na^{+}, the background $[Ca^{2+}]$ was held at a given level (ranging from 0 to 10 mmol l^{-1}) and the response

Table 1. Composition of artificial seawaters (ASW) used in the current study

	[NaCl]	[KCl]	[$CaCl_2$]	[$MgSO_4$]	[$MgCl_2$]	[Choline chloride]
ASW	460	10	10	25	25	–
Ca^{2+}-free ASW	460	10	–	25	25	20
Na^{+}-free ASW	–	10	10	25	25	460
Na^{+} and Ca^{2+}-free ASW	–	10	–	25	25	480
10‰ ASW	131.4	2.9	2.9	7.1	7.1	–
Ca^{2+}-free 10‰ ASW	131.4	2.9	–	7.1	7.1	5.8
$[Ca^{2+}]$ 10 mmol l^{-1} 10‰ ASW	131.4	2.9	10	7.1	7.1	–
Na^{+}-free 10 ‰ ASW	–	2.9	2.9	7.1	7.1	131.4
$[Na^{+}]$ 460 mmol l^{-1} 10‰ ASW	460	2.9	2.9	7.1	7.1	–

All values are shown as mmol l^{-1}. The pH of stock solutions was adjusted to 8.0–8.2 with 0.1 mol l^{-1} NaOH (or 0.1 mol l^{-1} KOH for Na^{+}-free solutions). Ranges of concentrations were made by appropriate mixing of the solutions.

to an increase in $[Na^+]$ from 0 to 460 mmol l^{-1} was recorded. These experiments were only carried out in fish adapted to 35‰ seawater.

The ranges of concentrations of the three amino acids were prepared with the same water perfusing the olfactory epithelium (35‰ or 10‰ ASW as appropriate). To assess the absence of either Ca^{2+} or Na^+ on the olfactory responses to amino acids, they were made up in Ca^{2+}-free ASW or Na^+-free ASW (35‰ or 10‰ as appropriate); the same water was used as background.

Data normalisation and statistical analysis

The amplitude of each integrated EEG response was blank subtracted (using the appropriate blank solution). For the $[Ca^{2+}]$, $[Na^+]$ and amino acid concentration/response curves, integrated response amplitudes were normalised to that of 10^{-3} mol l^{-1} L-cysteine. The bulbar response to 10^{-3} mol l^{-1} L-cysteine (in 35‰ ASW or 10‰ ASW as appropriate) was checked at regular intervals throughout the recording period. Bulbar EEG responses to changes in external $[Ca^{2+}]$ were fitted to a three-parameter Hill equation as previously described (Hubbard et al., 2000) to obtain the apparent IC_{50} (concentration giving a 50% maximal response), Hill co-efficient and I_{max} (maximum response) values. Apparent IC_{50} and I_{max} values (log transformed) were compared using Student's *t*-test. For the effect of variations of external Na^+ or Ca^{2+} on olfactory sensitivity to these ions and amino acids, the integrated response amplitude in the absence of either ion was normalised to control responses recorded in the presence of both ions (at 35‰ or 10‰ as appropriate). Differences in responsiveness to amino acids and sodium between 35‰ and 10‰ were assessed by linear regression

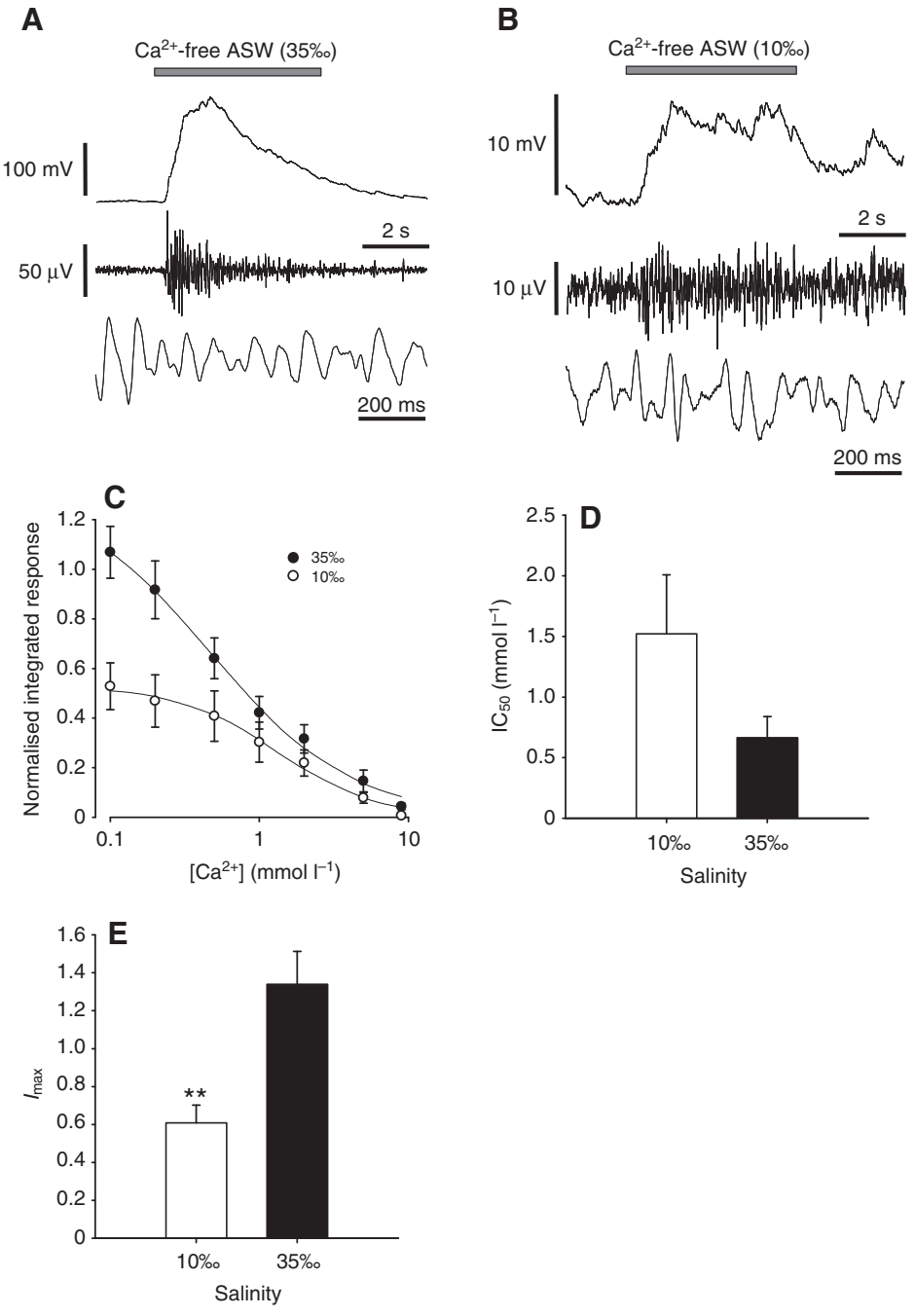

Fig. 1. Typical electro-encephalogram (EEG) recordings (middle trace) from the olfactory bulb of the sole in response to stimulation of the olfactory epithelium with Ca^{2+}-free artificial seawater (ASW) against a background of (A) $[Ca^{2+}]$ 10 mmol l^{-1} 10‰ ASW and (B) 35‰ ASW in fish adapted to 10‰ and 35‰ seawater, respectively (i.e. a reduction of $[Ca^{2+}]$ from 10 mmol l^{-1} to 0, independently of the salinity). Upper trace; integrated activity of raw signal (middle trace). Lower trace; expansion of the time-scale of the EEG during stimulation to show wave-form. (C) Semi-logarithmic plot of pooled data showing the olfactory responses to changes in external $[Ca^{2+}]$ of sole adapted to 10‰ (open circles) or 35‰ (closed circles). Data are shown as means ± s.e.m. (N=8). Apparent maximum response (I_{max}) (D) and concentration giving 50% maximal response (IC_{50}) (E) values calculated from Hill plots fitted to the data shown in C (N=8). $^{**}P<0.01$.

of log-transformed data (Hubbard et al., 2003; Velez et al., 2005) and comparing both the slopes and elevations of the regressions (Zar, 1996). For the effect of the absence of external Ca^{2+} and Na^{+} on bulbar responses to amino acids, the data were analysed by repeated-measures analysis of variance (ANOVA) followed by Dunnett's test (SigmaStat 2000, SPSS Science, Chicago, IL, USA). In all cases, $P<0.05$ was taken to represent statistical significance.

RESULTS

Olfactory responses to changes in environmental [Ca^{2+}]

Sole responded to a reduction of [Ca^{2+}] in ASW with an increase of EEG wave amplitude in the olfactory bulb (Fig. 1A,B); this increase was significantly higher in fish at 35‰ seawater than those adapted to 10‰ (Fig. 1C–E). At 10‰, the apparent IC_{50} was 1.52±0.49 mmol l^{-1} Ca^{2+} (mean ± s.e.m., N=8) with a Hill co-efficient of –1.8±0.5 and an I_{max} of 0.61±0.09. At 35‰, the olfactory sensitivity to reductions in environmental [Ca^{2+}] was slightly higher, as reflected by a lower IC_{50} of 0.66±0.17 mmol l^{-1} (mean ± s.e.m., N=8), a Hill coefficient of –1.3±0.2 and a higher I_{max} of 1.34±0.17. However, only the I_{max} was statistically different between 35‰ and 10‰ adapted fish (Fig. 1E). During stimulation with reductions in external [Ca^{2+}], EEG wave frequency in the olfactory bulb was similar to basal bulbar activity and there were no significant differences between 35‰ and 10‰ adapted fish (data not shown). Substitution of $CaCl_2$ with choline chloride did not affect the responses; neither changes in osmolality nor [Cl^{-}] are responsible for the observed responses (data not shown).

Olfactory sensitivity to changes in environmental [Na^{+}]

As with Ca^{2+}, during stimulation with Na^{+}, EEG wave frequency in the olfactory bulb was similar to basal bulbar activity, neither were there differences between 10‰ and 35‰ seawater. However, sole responded to increases of external [Na^{+}] with a marked increase in wave amplitude in the olfactory bulb (Fig. 2A,B). This increase in amplitude was significantly higher in fish adapted to 10‰ seawater than in those kept at 35‰ (Fig. 2C). In contrast to the responses to changes in [Ca^{2+}], bulbar responses were only seen to increases in external [Na^{+}]; reduction of external [Na^{+}] failed to evoke any response at the two salinities tested (Fig. 2D). As the bulbar responses to increases of external [Na^{+}] showed no sign of reaching a maximum, even at 460 mmol l^{-1}, concentration/response curves could not be fitted to a conventional three-parameter Hill equation. The concentration/response curves to changes in [Na^{+}] at 35‰ and 10‰ ASW were, nevertheless, statistically different in both the slopes and elevations of the regressions (Fig. 2C). Substitution of NaCl with choline chloride did not evoke any response; neither changes in osmolality nor [Cl^{-}] were responsible for the observed responses (data not shown).

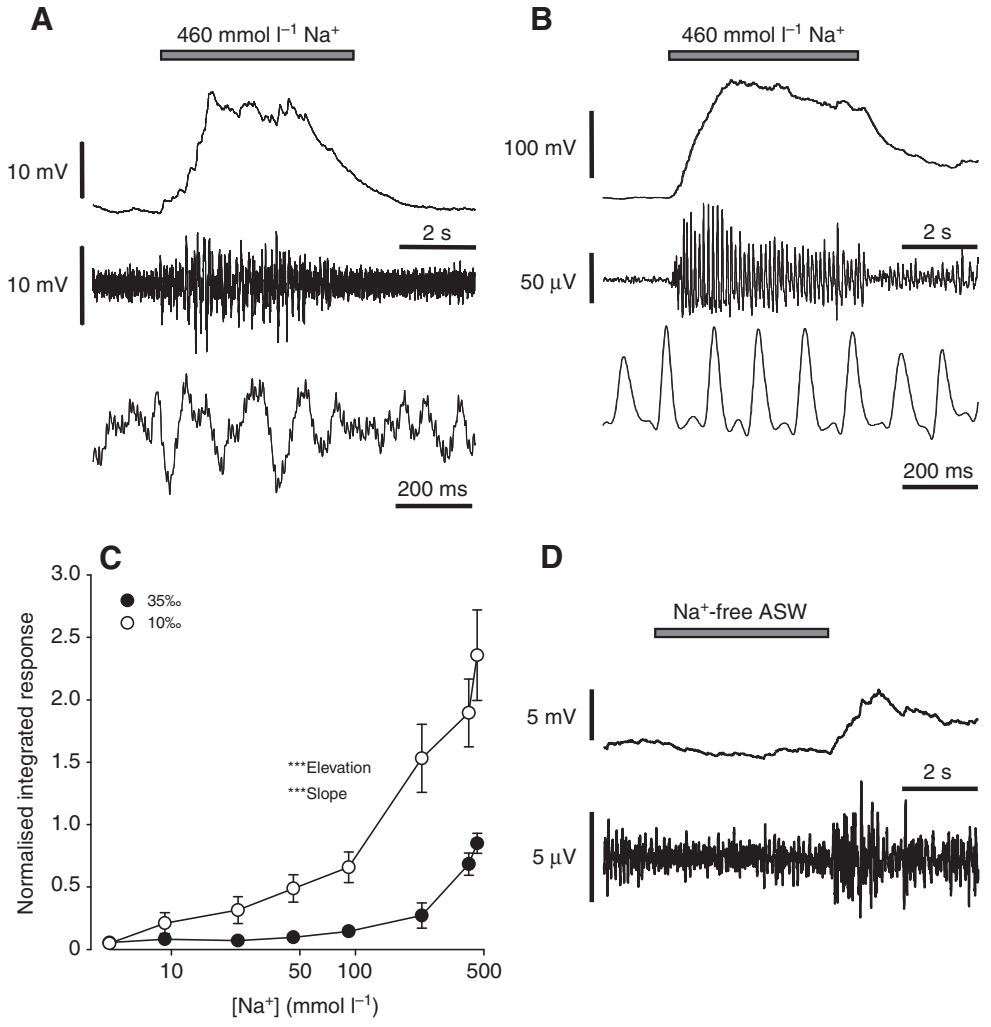

Fig. 2. Typical electro-encephalogram (EEG) recordings (middle trace) from the olfactory bulb of sole in response to an increase in [Na^{+}] of 460 mmol l^{-1} artificial seawater (ASW) against a background of (A) 10‰ ASW (Na^{+}-free) and (B) 35‰ ASW (Na^{+}-free) in fish adapted to 10‰ and 35‰ seawater, respectively. Upper trace; integrated activity of raw signal (middle trace). Lower trace; expansion of the time-scale of the EEG during stimulation to show wave-form. (C) Semi-logarithmic plot of pooled data showing the olfactory responses to increases in external [Na^{+}] of sole adapted to 10‰ (open circles) and 35‰ (closed circles) seawater. Note that the responses were recorded against a background of Na^{+}-free ASW (10‰ or 35‰ as appropriate). Data are shown as means ± s.e.m. (N=8). ***$P<0.001$, statistically significant differences were found between the slope and elevation of the regression curves fitted to the data at the two different salinities. (D) Typical EEG recording (lower trace) and integrated signal (upper trace) from the olfactory bulb of sole in response to a decrease in [Na^{+}] from 460 mmol l^{-1} to nominally zero (Na^{+}-free ASW). Note the lack of response to the decrease in [Na^{+}] but the response to return to ASW at the end of the stimulus (recorded in a fish adapted to 35‰ seawater).

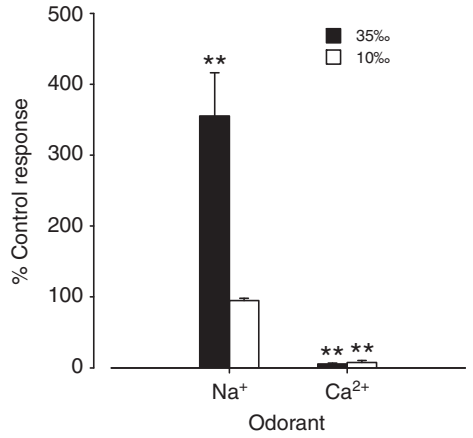

Fig. 3. Effect of the absence of external Ca^{2+} on the olfactory bulbar response to Na^+ (0 to 460 mmol l^{-1}; left bars) and the absence of external Na^+ on the olfactory bulbar response to Ca^{2+} (10 mmol l^{-1} to 0; right bars) recorded from sole adapted to 35‰ (closed bars) or 10‰ (open bars) seawater. Data are shown as means ± s.e.m. (N=6). $^{**}P$<0.01 compared with control (with Na^+-free artificial seawater (ASW) and Na^+-free 10‰ ASW as background, in the case responses to Na^+, and ASW or 10 mmol l^{-1} [Ca^{2+}] 10‰ ASW as background, in the case of responses to Ca^{2+}). Note that the absence of external Na^+ attenuated the bulbar response to changes in [Ca^{2+}], independent of salinity whereas the bulbar responses to changes in [Na^+] are increases in the absence of Ca^{2+} in fish adapted to 35‰ but not 10‰ seawater.

Effect of absence of external Ca^{2+} and Na^+ on olfactory sensitivity

At 10‰, the absence of external Ca^{2+} did not alter bulbar responses to increases in external [Na^+] (Fig. 3). However, at 35‰, bulbar responses to increases in external [Na^+] increased more than threefold in the absence of external Ca^{2+} compared with control. Conversely, the absence of external Na^+ completely attenuated the bulbar response to decreases in external [Ca^{2+}] at both salinities (Fig. 3). The bulbar responses to reductions (10 mmol l^{-1} to 0) of external [Ca^{2+}] at different background (i.e. continuous) [Na^+] levels were severely blunted when the external [Na^+] was below 46 mmol l^{-1}. Above this [Na^+], the amplitude of bulbar responses to decreases in external [Ca^{2+}] increased exponentially with background [Na^+] (Fig. 4A). Conversely, the dependence of bulbar responses to increases in external [Na^+] (from 0 to 460 mmol l^{-1}) showed the opposite trend. At low background [Ca^{2+}] levels (0.1–0.2 mmol l^{-1}), responses to Na^+ were similar to those in the absence of external Ca^{2+} (Fig. 4B). However, at a background [Ca^{2+}] of 0.5 mmol l^{-1} or more, the bulbar responses to increases in external [Na^+] became progressively smaller. Nevertheless, bulbar responses to Na^+ were never completely attenuated; even at a background [Ca^{2+}] of 9 mmol l^{-1}, appreciable responses to increases in external [Na^+] were recorded.

Olfactory sensitivity to amino acids

Stimulation of the olfactory epithelium with L-cysteine evoked an increase of EEG wave amplitude in the olfactory bulb in a concentration-dependent manner (Fig. 5A). However, there was no apparent effect of salinity on the concentration/response curve to L-cysteine (Fig. 5C). Bulbar EEG wave frequency in response to stimulation with L-cysteine was similar to basal activity with no differences between 35‰ and 10‰ adapted fish, and the EEG waveforms evoked by L-cysteine were similar to those evoked by changes in [Ca^{2+}] and [Na^+]. Similarly, there were no statistically significant effects of salinity on the olfactory sensitivity to amino acids glycine and L-phenylalanine (Figs 6 and 7). Bulbar responses to amino acids in both salinities did not reach a maximum, even at 1.0 mmol l^{-1}.

The effects of the absence of external Ca^{2+} or Na^+ on olfactory sensitivity to amino acids were similar for all three amino acids tested (Fig. 8). The bulbar responses to amino acids at both salinities were not altered by the absence of external Ca^{2+} whereas the absence of Na^+ markedly decreased the amplitude of bulbar responses to all three amino acids (to about 20% of control) at 35‰ but not at 10‰.

DISCUSSION

Olfactory sensitivity to Ca^{2+} and Na^+

The current study shows that the olfactory system of the sole responds to decreases in environmental [Ca^{2+}] and increases in environmental [Na^+]. Both ions evoked large-amplitude, low-frequency (~10 Hz) wave activity in the olfactory bulb, similar to that previously described in goldfish (Hubbard and Canário, 2007) and similar to that evoked by 'conventional' odorants, such as amino acids, in the sole. The response to decreases in [Ca^{2+}] was also similar to, albeit slightly more sensitive than, that of the seabream in terms of the apparent IC_{50} (seabream; 1.7 mmol l^{-1}, sole; 0.7 mmol l^{-1}) and amplitude (Hubbard et al., 2000); this may be due to the different recording methods used (i.e. multi-unit recording from the olfactory nerve and bulbar EEG, respectively). However, continual exposure of the olfactory epithelium of the seabream to Ca^{2+}-free ASW caused a decrease in the apparent IC_{50} to 0.5 mmol l^{-1} (i.e. increased sensitivity); in the sole, adaptation to 10‰ seawater reduced the sensitivity (apparent IC_{50}; 1.5 mmol l^{-1}) and amplitude

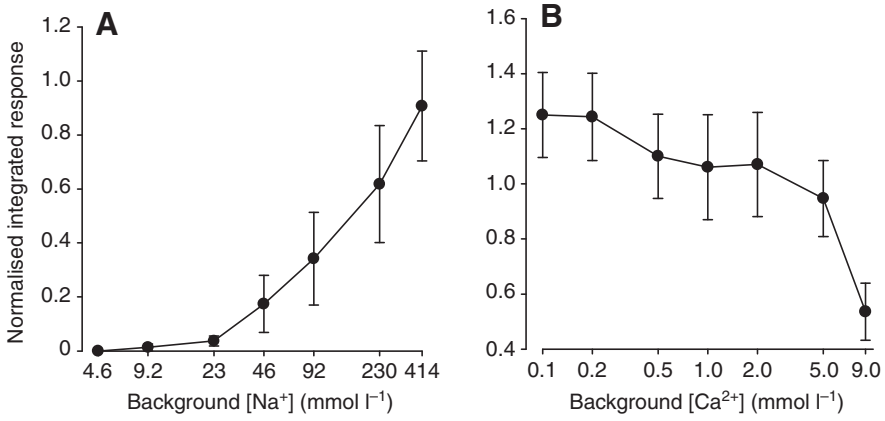

Fig. 4. (A) Semi-logarithmic plot showing the effect of increasing background [Na^+] on the olfactory bulbar response to changes in external [Ca^{2+}] (10 mmol l^{-1} to 0) in the sole (N=4). (B) Semi-logarithmic plot of pooled data showing the effect of increasing background [Ca^{2+}] on the olfactory bulbar response to changes in external [Na^+] (0 to 460 mmol l^{-1}). Data are shown as means ± s.e.m. (N=4). Both experiments were recorded from sole adapted to 35‰ seawater. Note that increasing background [Na^+] increases the olfactory response to Ca^{2+} whereas increasing background [Ca^{2+}] decreases the response to Na^+.

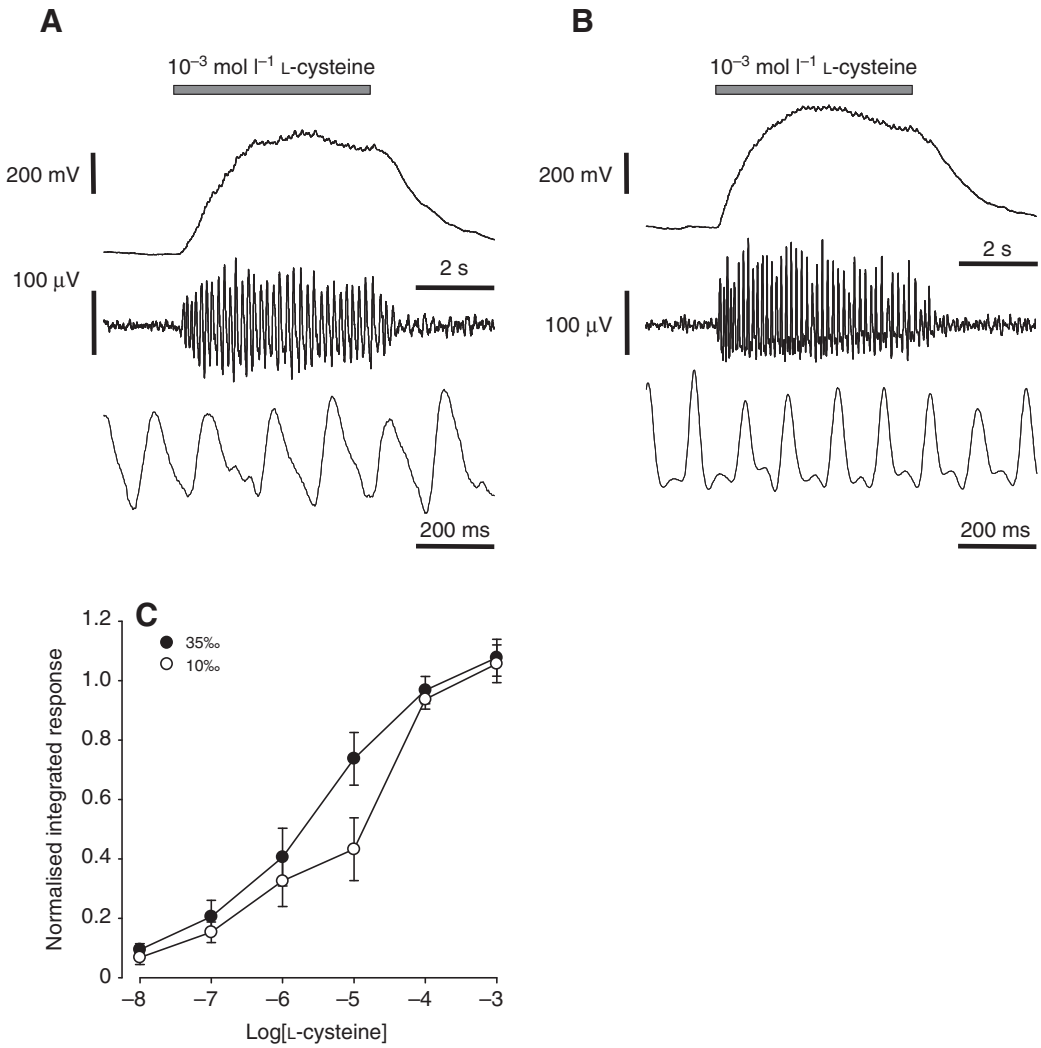

Fig. 5. Typical EEG recordings (middle trace) from the olfactory bulb in response to 10^{-3} mol l^{-1} L-cysteine from sole adapted to (A) 10‰ and (B) 35‰ seawater. Upper trace; integrated activity of raw signal (middle trace), lower trace; expansion of the time-scale of the EEG to show wave-form. (C) Semi-logarithmic plot of pooled data showing the olfactory responses of sole to 10^{-3} mol l^{-1} L-cysteine in 10‰ artificial seawater (ASW) (open circles) and ASW (35‰; closed circles) in fish adapted to 10‰ and 35‰ seawater, respectively. Data are shown as means ± s.e.m. (N=6). No statistically significant differences were found between the slope (P>0.50) or elevation (0.05<P<0.10) of the regression curves fitted to the data at the two different salinities.

of the response. This discrepancy may be explained by the effect of Na^+ on the olfactory response to Ca^{2+}, because the olfactory response to changes in environmental [Ca^{2+}] is decreased by low external [Na^+]. The olfactory response to reductions of external [Ca^{2+}] was recorded against a background [Na^+] of 460 mmol l^{-1} in the seabream (Hubbard et al., 2000) whereas the olfactory response of sole adapted to 10‰ seawater was recorded against a background [Na^+] of 131 mmol l^{-1}. Further work will clarify whether this change in sensitivity reflects an active adaptation on the part of the olfactory system to lower environmental [Ca^{2+}] (i.e. the sensitivity to a given stimulus is 'tuned' to its prevailing intensity) or if it can be explained simply as a direct result of the lower [Na^+] of 10‰ seawater (see below).

The olfactory system of sole responded to increases in environmental [Na^+] in a similar way to freshwater-reared salmon and goldfish (Bodznick, 1978; Hubbard et al., 2002). As far as the authors are aware, this is the first time that an olfactory response to changes in environmental [Na^+] has been documented in a marine fish. In contrast to the response to changes in external [Ca^{2+}], the olfactory system did not respond to decreases in external [Na^+] but only to increases. The functional significance of this observation remains unclear. Nevertheless, the sensitivity to changes in [Na^+] was increased by adaptation to low salinity (10‰). This contrasts directly with the effect of increasing [Ca^{2+}] on the olfactory sensitivity to Na^+ in the goldfish (Hubbard and Canário, 2007), where increasing the background [Ca^+] reduced the sensitivity to Na^+. Clearly, the ion levels of seawater and freshwater are very different, this discrepancy between the olfactory responses of marine and freshwater fish probably depends on the exact mechanisms involved (see below).

Detection mechanisms for Ca^{2+} and Na^+

It has been suggested that the olfactory sensitivity of fish to Ca^{2+} is mediated by a Ca^{2+}-sensing receptor (Ca^{2+}-SR) (Hubbard et al., 2002), similar to that cloned from bovine parathyroid gland (Brown et al., 1993); this receptor is present in the olfactory epithelia of several fish (Hubbard et al., 2002; Nearing et al., 2002). Nevertheless, calcium is not the only ligand of the Ca^{2+}-SR; it also binds other divalent and trivalent cations, including Mg^{2+} (present in seawater at ~50 mmol l^{-1}), although its affinity for Mg^{2+} is much lower than that for Ca^{2+} (Chang and Shoback, 2004). Consistent with this, the olfactory sensitivity of goldfish to changes in external [Mg^{2+}] is correspondingly less (Hubbard et al., 2002) and the sensitivity to reductions in [Ca^{2+}] in the seabream remains acute despite the continued presence of 50 mmol l^{-1} Mg^{2+} (Hubbard et al., 2000). Olfactory sensitivity to Mg^{2+} and the effect of Mg^{2+} on the sensitivities to Ca^{2+} and Na^+ were not investigated in the present study.

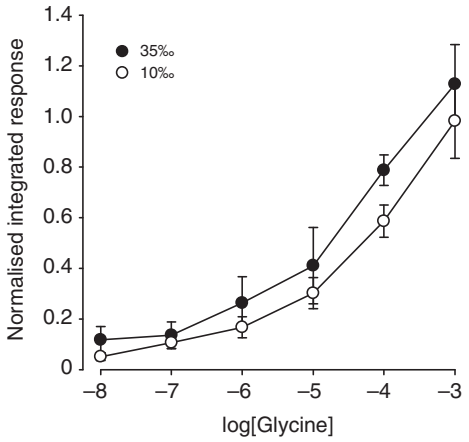

Fig. 6. Semi-logarithmic plot of pooled data (N=6) showing the olfactory responses of sole to 10^{-3} mol l^{-1} glycine in 10‰ artificial seawater (ASW) (open circles) and ASW (35‰; closed circles) in fish adapted to 10‰ and 35‰ seawater, respectively. Data are shown as means ± s.e.m. (N=6). No statistically significant differences were found between the slope (P>0.50) or elevation (0.10<P<0.20) of the regression curves fitted to the data at the two different salinities.

The affinity for Ca^{2+} of the mammalian Ca^{2+}-SR is reduced by elevated extracellular [Na^+] (Quinn et al., 1998) possibly due to 'shielding' of the Ca^{2+}-binding site by Na^+ ions (Loretz, 2008). Similarly, the olfactory sensitivity of the goldfish to changes in [Ca^{2+}] is reduced by increasing environmental [Na^+] (Hubbard and Canário, 2007). However, in the sole, increased environmental [Na^+] increased the olfactory sensitivity to changes in [Ca^{2+}]. This may be due to a reliance on external Na^+ ions of the transduction pathway for the sensitivity to external Ca^{2+}. To resolve this, investigation of the transduction pathway(s) involved is necessary. Alternatively, the olfactory Ca^{2+}-SR of both marine and freshwater teleosts may be different from the mammalian Ca^{2+}-SR. Extracellular pH also affects the affinity of the Ca^{2+}-SR to Ca^{2+} (Quinn et al., 2004); how changes in environmental pH affect olfactory sensitivity to Ca^{2+} awaits investigation.

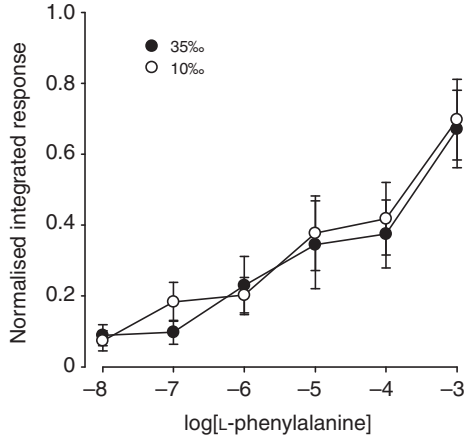

Fig. 7. Semi-logarithmic plot of pooled data (N=6) showing the olfactory responses of sole to 10^{-3} mol l^{-1} L-phenylalanine in 10‰ artificial seawater (ASW) (open circles) and ASW (35‰; closed circles) in fish adapted to 10‰ and 35‰ seawater, respectively. Data are shown as means ± s.e.m. (N=6). No statistically significant differences were found between slope (P>0.50) or elevation (P>0.50) of the regression curves fitted to the data at the two different salinities.

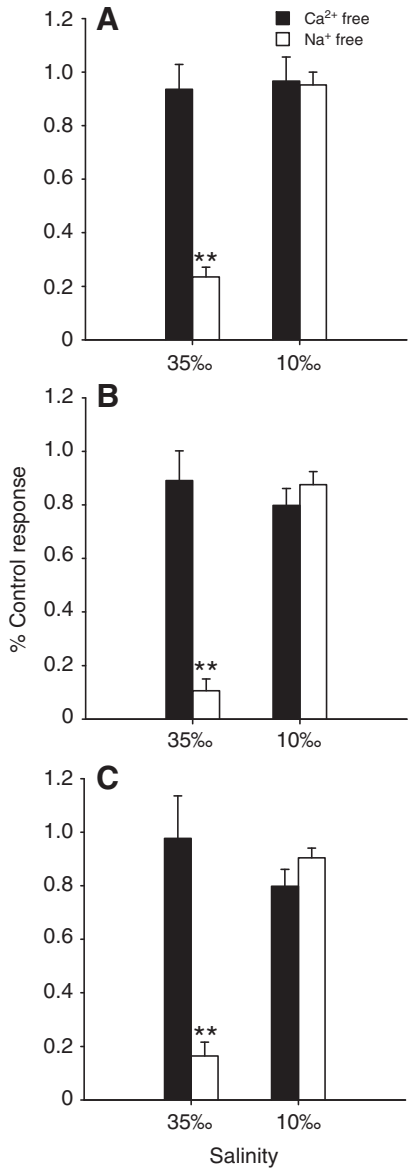

Fig. 8. Effect of the absence of external Ca^{2+} (closed bars) or Na^+ (open bars) on the olfactory bulbar responses to 10^{-3} mol l^{-1} (A) L-cysteine, (B) glycine and (C) L-phenylalanine stimulating the olfactory epithelium in fish adapted to 35‰ or 10‰ seawater. Data are shown as means ± s.e.m. (N=6). **P<0.01 compared with control. Note that the amplitude of responses is reduced by the absence of Na^+ in fish adapted to 35‰ seawater only.

In mammals, activation of the Ca^{2+}-SR elicits a variety of G-protein-mediated intracellular signals, including activation of phospholipase C (PLC), cytosolic phospholipase A_2 ($cPLA_2$), phosphatidyl inositol 4-kinase (PI_4K), mitogen-activated (MAP) kinases (extracellular-signal-regulated kinase, ERK, and Jun amino-terminal kinase, JNK) and the inhibition of adenylate cyclase (AC) (Breitwieser, 2008; Hofer and Brown, 2003). Assuming that responses to Ca^{2+} are mediated by a Ca^{2+}-SR, a possible explanation for the olfactory sensitivity of sole to decreases of [Ca^{2+}] is that, in seawater, there is a high level of receptor occupancy and, consequently, AC activity is inhibited. As the external [Ca^{2+}]

decreases, Ca^{2+} is released from the receptor and AC becomes activated, leading to olfactory neurone depolarisation. In contrast to the goldfish (Hubbard and Canário, 2007), olfactory responses to changes in $[Ca^{2+}]$ in the sole are increased by increasing external $[Na^+]$; in the absence of external Na^+, olfactory sensitivity to Ca^{2+} is completely abolished. Thus, in the sole, olfactory sensitivity to Ca^{2+} depends on the presence of external Na^+.

Na^+ has a negative effect on the activation of mammalian Ca^{2+}-SR by Ca^{2+}, possibly by competition with Ca^{2+} ions for the Ca^{2+}-binding site (Quinn et al., 1998). Thus, decreasing $[Na^+]$ leads to a higher activation of the Ca^{2+}-SR by a given increase in $[Ca^{2+}]$ and a subsequent increase in sensitivity. This may explain why increasing $[Na^+]$ attenuates olfactory sensitivity to changes in $[Ca^{2+}]$ in the goldfish (Hubbard and Canário, 2007). Given that the olfactory system of the sole responds to decreases in external $[Ca^{2+}]$ and, presumably, to disassociation of Ca^{2+} ions from the Ca^{2+}-SR, the higher $[Na^+]$ of 35‰ seawater may reduce the affinity of the receptor for Ca^{2+} ions and make this dissociation more likely, thereby increasing olfactory sensitivity to changes in $[Ca^{2+}]$. Activation of non-selective cation channels by Ca^{2+}-SR has been described in hippocampal pyramidal cells (Ye et al., 1996a; Ye et al., 1996b). This could explain the abolishment of olfactory response to Ca^{2+} in the absence of Na^+ and the higher olfactory sensitivity to Ca^{2+} recorded in sole adapted to 35‰ compared with those adapted to 10‰ seawater; external Na^+ ions may be necessary for the transduction of the response to Ca^{2+}.

The sole has olfactory sensitivity to increases in external $[Na^+]$ but to decreases in external $[Ca^{2+}]$; however, the olfactory responses to changes in $[Na^+]$ seem to be mediated by a different mechanism from those to changes in $[Ca^{2+}]$. Furthermore, the concentration/response curve to changes in external $[Ca^{2+}]$ is sigmoidal, suggesting a receptor-mediated mechanism whereas the concentration/response curve to $[Na^+]$ does not reach a maximum (within the range of concentrations tested), suggesting that responses to Na^+ could be channel-mediated (Hubbard and Canário, 2007). In addition, if both Ca^{2+} and Na^+ responses were due to activation of the same cells, the absence of Ca^{2+} would depolarise these neurones; thus, inhibiting responses to Na^+ whereas decreases of background $[Ca^{2+}]$ have the opposite effect on olfactory sensitivity to Na^+. Furthermore, high external $[Na^+]$ would inhibit sensitivity to Ca^{2+}. In sole, however, the absence of external Ca^{2+} increases olfactory bulbar responses to Na^+ whereas responses to Ca^{2+} are attenuated by low external $[Na^+]$. We suggest that olfactory responses to Na^+ are mediated by a channel that allows the influx of Na^+ ions and, possibly, other cations. Ca^{2+} ions may also enter this channel but, instead of entering the cell, remain in the pore and block it. The existence of Na^+-activated non-selective cation channels has been shown in the olfactory receptor neurones of lobsters (Zhainazarov and Ache, 1998; Zhainazarov et al., 1998); these Na^+-activated channels are (as are other Na^+-activated channels from different systems) blocked by Ca^{2+} (Armstrong and Cota, 1999).

Olfactory sensitivity to amino acids

Fish, in general, have high olfactory sensitivity to amino acids (Hara, 1994; Michel, 2006) and the sole is no exception (Velez et al., 2005; Velez et al., 2007b). The slightly higher sensitivity of sole to amino acids reported in the current study may simply be a reflection of the different method used; previous studies used the EOG, which is likely to underestimate the true sensitivity due to the shunting effect of seawater (Velez et al., 2005). Recording from the olfactory bulb (EEG) eliminates this problem and allows a direct comparison of sensitivity at different salinities. Sole are able to maintain olfactory sensitivity to amino acids independently of external salinity; no differences were seen between the bulbar responses to L-cysteine, L-phenylalanine and glycine at 35‰ and 10‰ with thresholds of detection around 10^{-7}–10^{-8} mol l^{-1} in all cases. This is consistent with previous studies with euryhaline salmonids (Shoji et al., 1994; Shoji et al., 1996) and would be expected for a fish, such as sole, that often penetrates estuaries in order to feed (Cabral, 2000). Nevertheless, the olfactory transduction mechanism(s) must somehow adapt to the lower availability of Na^+ in estuarine water; at 35‰, the olfactory response to amino acids is dependent on external Na^+ but is independent of Na^+ at 10‰. Whether this is achieved by regulating the ionic component of the mucus layer overlying the olfactory epithelium as in mammals (Schild and Restrepo, 1998) or by shifting the transduction pathway to use extracellular (rather than external) Na^+ is not yet known. In the seabream, exposure of the olfactory epithelium to Ca^{2+}-free ASW caused a temporary diminution of the response to L-serine, suggesting some reliance of the transduction mechanism on external Ca^{2+} which, again, could be overcome (Hubbard et al., 2000). Olfactory responses of the sole to amino acids are apparently independent of external Ca^{2+}. This may reflect different ion channels (Na^+-selective or Ca^{2+}-selective) being involved in the transduction mechanisms of the different species. The olfactory transduction pathways in sole are currently under investigation.

Possible environmental implications

For a fish that enters waters of reduced salinity to feed, such as the sole, it would be important to maintain olfactory sensitivity to amino acids. The current study suggests that sole are able to do so but that this requires some modulation of the olfactory system in that reliance on external Na^+ is reduced as it adapts to reduced salinity. How this is achieved will be the subject of further studies. Sensitivity to reductions in environmental $[Ca^{2+}]$, however, may serve as a proxy for reductions in salinity (Hubbard et al., 2000; Nearing et al., 2002), either to warn the fish that it may be reaching the limit of its osmoregulatory tolerance and/or regulate the osmoregulatory mechanisms themselves. As reduced salinity reduces the sensitivity to reductions in environmental $[Ca^{2+}]$, the sole may then seek out water of higher salinity by using its sensitivity to increases in environmental $[Na^+]$. These hypotheses wait further testing.

Conclusion

The olfactory system of sole responds to decreases in environmental $[Ca^{2+}]$ but increases in $[Na^+]$. Sensitivity to Ca^{2+} is higher at 35‰ but that to Na^+ is higher in 10‰ seawater. Although the sensitivities to the two ions are mediated by apparently different mechanisms, changes in the background levels of one affect the sensitivity to the other; sensitivity to changes in environmental $[Ca^{2+}]$ are dependent on external Na^+ whereas olfactory responses to Na^+ are reduced by increasing $[Ca^{2+}]$. Olfactory sensitivity to amino acids is unaffected by reduced salinity. However, at 35‰, the absence of external Na^+ (but not Ca^{2+}) markedly reduces the response to amino acids. At 10‰ the response to amino acids depends on neither external Ca^{2+} nor Na^+. This suggests that the olfactory system must adapt to lower salinities by reducing the dependence on environmental Na^+.

LIST OF ABBREVIATIONS

AC	adenylate cyclase
ANOVA	analysis of variance
ASW	artificial seawater

Ca^{2+}-SR	calcium-sensing receptor
$cPLA_2$	cytosolic phospholipase A_2
EEG	electro-encephalogram
EOG	electro-olfactogram
ERK	extracellular-signal-regulated kinase
IC_{50}	concentration giving a 50% maximal response
I_{max}	maximum response
JNK	Jun amino-terminal kinase
MAP kinase	mitogen-activated protein kinase
PI_4K	phosphatidyl inositol 4-kinase
PLC	phospholipase C

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REFERENCES

Armstrong, C. M. and Cota, G. (1999). Calcium block of Na^+ channels and its effect on closing rate. *Proc. Natl. Acad. Sci. USA* **96**, 4154-4157.

Bayarri, M. J., Muñoz-Cueto, J. A., López-Olmeda, J. F., Vera, L. M., Rol de Lama, M. A., Madrid, J. A. and Sánchez-Vázquez, F. J. (2004). Daily locomotor activity and melatonin rhythms in Senegal sole (*Solea senegalensis*). *Physiol. Behav.* **81**, 577-583.

Bodznick, D. (1978). Calcium ion: an odorant for natural water discriminations and the migratory behavior of sockeye salmon. *J. Comp. Physiol. A* **127**, 157-166.

Breitwieser, G. E. (2008). Extracellular calcium as an integrator of tissue function. *Int. J. Biochem. Cell Biol.* **40**, 1467-1480.

Brierley, M. J., Ashworth, A. J., Banks, J. R., Balment, R. J. and McCrohan, C. R. (2001). Bursting properties of caudal neurosecretory cells in the flounder *Platichthys flesus*, *in vitro*. *J. Exp. Biol.* **204**, 2733-2739.

Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J. and Hebert, S. C. (1993). Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature* **366**, 575-580.

Cabral, H. N. (2000). Comparative feeding ecology of sympatric *Solea solea* and *S. senegalensis*, within nursery areas of the Tagus estuary, Portugal. *J. Fish Biol.* **57**, 1550-1562.

Chang, W. and Shoback, D. (2004). Extracellular Ca^{2+}-sensing receceptors-an overview. *Cell Calcium* **35**, 183-196.

Dinis, M. T., Ribeiro, L., Soares, F. and Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture* **176**, 27-38.

Hara, T. J. (1994). The diversity of chemical stimulation in fish olfaction and gustation. *Rev. Fish Biol. Fish.* **4**, 1-35.

Hofer, A. M. and Brown, E. M. (2003). Extracellular calcium sensing and signalling. *Nat. Rev. Mol. Cell Biol.* **4**, 530-538.

Hubbard, P. C. and Canário, A. V. M. (2007). Evidence that olfactory sensitivities to calcium and sodium are mediated by different mechanisms in the goldfish *Carassius auratus*. *Neurosci. Lett.* **414**, 90-93.

Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2000). Olfactory sensitivity to changes in environmental [Ca^{2+}] in the marine teleost *Sparus aurata*. *J. Exp. Biol.* **203**, 3821-3829.

Hubbard, P. C., Ingleton, P. M., Bendell, L. A., Barata, E. N. and Canário, A. V. M. (2002). Olfactory sensitivity to changes in environmental [Ca^{2+}] in the freshwater teleost *Carassius auratus*: an olfactory role for the Ca^{2+}-sensing receptor? *J. Exp. Biol.* **205**, 2755-2764.

Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2003). Olfactory sensitivity to catecholamines and their metabolites in the goldfish. *Chem. Senses* **28**, 207-218.

Loretz, C. A. (2008). Extracellular calcium-sensing receptors in fishes. *Comp. Biochem. Physiol.* **149A**, 225-245.

Menini, A. (1999). Calcium signalling and regulation in olfactory neurons. *Curr. Opin. Neurobiol.* **9**, 419-426.

Michel, W. C. (2006). Chemoreception. In *The Physiology of Fishes* (ed. D. H. Evans and J. B. Claiborne), pp. 471-497. Boca Raton, FL: CRC Press.

Nakamura, T. (2000). Cellular and molecular constituents of olfactory sensation in vertebrates. *Comp. Biochem. Physiol.* **126A**, 17-32.

Nearing, J., Betka, M., Quinn, S., Hentschel, H., Elger, M., Baum, M., Bai, M., Chattopadyhay, N., Brown, E. M., Hebert, S. C. et al. (2002). Polyvalent cation receptor proteins (CaRs) are salinity sensors in fish. *Proc. Natl. Acad. Sci. USA* **99**, 9231-9236.

Quinn, S. J., Kifor, O., Trivedi, S., Diaz, R., Vassilev, P. and Brown, E. (1998). Sodium and ionic strength sensing by the calcium receptor. *J. Biol. Chem.* **273**, 19579-19586.

Quinn, S. J., Bai, M. and Brown, E. M. (2004). pH sensing by the calcium-sensing receptor. *J. Biol. Chem.* **279**, 37241-37249.

Schild, D. and Restrepo, D. (1998). Transduction mechanisms in vertebrate olfactory receptor cells. *Physiol. Rev.* **78**, 429-466.

Shoji, T., Fujita, K. I., Ban, M., Hiroi, O., Ueda, H. and Kurihara, K. (1994). Olfactory responses of chum salmon to amino acids are independent of large differences in salt concentrations between fresh and sea water. *Chem. Senses* **19**, 609-615.

Shoji, T., Fujita, K. I., Furihata, E. and Kurihara, K. (1996). Olfactory responses of a euryhaline fish, the rainbow trout: adaptation of olfactory receptors to sea water and salt-dependence of their responses to amino-acids. *J. Exp. Biol.* **199**, 303-310.

Velez, Z., Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2005). Evidence for functional asymmetry in the olfactory system of the Senegalese sole (*Solea sensgalensis*). *Physiol. Biochem. Zool.* **78**, 756-765.

Velez, Z., Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2007a). Differential detection of conspecific-derived odorants by the two olfactory epithelia of the Senegalese sole (*Solea senegalensis*). *Gen. Comp. Endocrinol.* **153**, 418-425.

Velez, Z., Hubbard, P. C., Hardege, J. D., Barata, E. N. and Canário, A. V. M. (2007b). The contribution of amino acids to the odour of a prey species in the Senegalese sole (*Solea senegalensis*). *Aquaculture* **265**, 336-342.

Velez, Z., Hubbard, P. C., Welham, K., Hardege, J. D., Barata, E. N. and Canário, A. V. M. (2009). Identification, release and olfactory detection of bile acids in the intestinal fluid of the Senegalese sole (*Solea senegalensis*) *J. Comp. Physiol. A* **195**, 691-698.

Ye, C., Kanazirska, M., Quinn, S., Brown, E. E. and Vassilev, P. M. (1996a). Modulation by polycationic Ca^{2+}-sensing receptor agonists of nonselective cation channels in rat hippocampal neurons. *Biochem. Biophys. Res. Commun.* **224**, 271-280.

Ye, C., Rogers, K., Bai, M., Quinn, S. J., Brown, E. M. and Vassilev, P. M. (1996b). Agonists of the Ca^{2+}-sensing receptor (CaR) activate nonselective cation channels in HEK293 cells stably transfected with the human CaR. *Biochem. Biophys. Res. Commun.* **226**, 572-579.

Zar, J. H. (1996). *Biostatistical Analysis*. Upper Saddle River, NJ: Pearson Higher Education.

Zhainazarov, A. B. and Ache, B. W. (1998). Na^+-gated nonselective cation channel from lobster olfactory projection neurons. *J. Neurophysiol.* **80**, 3387-3391.

Zhainazarov, A. B., Doolin, R. E. and Ache, B. W. (1998). Sodium-gated cation channel implicated in the activation of lobster olfactory receptor neurons. *J. Neurophysiol.* **79**, 1349-1359.