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RESEARCH ARTICLE

Environmental salinity modulates olfactory sensitivity in the euryhaline European seabass, *Dicentrarchus labrax*, acclimated to seawater and brackish water

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ABSTRACT

The olfactory epithelium of fish is – of necessity – in intimate contact with the surrounding water. In euryhaline fish, movement from seawater to freshwater (and vice versa) exposes the epithelium to massive changes in salinity and ionic concentrations. How does the olfactory system function in the face of such changes? The current study compared olfactory sensitivity in seawater- (35‰) and brackish water-adapted seabass (5‰) using extracellular multi-unit recording from the olfactory nerve. Seawater-adapted bass had higher olfactory sensitivity to amino acid odorants when delivered in seawater than in freshwater. Conversely, brackish water-adapted bass had largely similar sensitivities to the same odorants when delivered in seawater or freshwater, although sensitivity was still slightly higher in seawater. The olfactory system of seawater-adapted bass was sensitive to decreases in external [Ca^{2+}], whereas brackish water-adapted bass responded to increases in [Ca^{2+}]; both seawater- and brackish water-adapted bass responded to increases in external [Na^{+}] but the sensitivity was markedly higher in brackish water-adapted bass. In seawater-adapted bass, olfactory sensitivity to L-alanine depended on external Ca^{2+} ions, but not Na^{+}; brackish water-adapted bass did respond to L-alanine in the absence of Ca^{2+}, albeit with lower sensitivity, whereas sensitivity was unaffected by removal of Na^{+} ions. A possible adaptation of the olfactory epithelium was the higher number of mucous cells in brackish water-adapted bass. The olfactory system of seabass is able to adapt to low salinities, but this is not immediate; further studies are needed to identify the processes involved.

KEY WORDS: Olfaction, Salinity, Osmoregulation, Calcium, Sodium, Seabass, Amino acids

INTRODUCTION

Living organisms use chemosensory systems (e.g. olfaction and taste) for many vital processes. For aquatic animals, the olfactory system is essential, as it can detect a wide range of small molecular weight water-soluble compounds which elicit crucial behaviours such as feeding, reproduction, social interaction and predator avoidance (Hamdani and Døving, 2007; Laberge and Hara, 2001; Zeiske et al., 1992). This is especially important in nocturnal feeders or those living in turbid waters, which rely almost exclusively on this function. Therefore, any disruption to this system could reduce their capacity to capture prey, mate and avoid predators, and ultimately reduce reproduction and survival.

The olfactory system of fish is in intimate contact with the surrounding water. Teleosts have two nasal cavities, one on each side of the head near the snout (Kermen et al., 2013). Although there are some exceptions, generally, each nasal cavity is composed of an anterior nare, through which water enters the nose, and a posterior exhalent nare. The olfactory epithelium lies between these two nostrils (Kermen et al., 2013); odorants are detected upon interaction with olfactory receptors (ORs) in the olfactory sensory neurons (OSNs) within the olfactory epithelium. In fish, as in mammals, the detection of odorants by olfactory sensory neurons is mediated by different families of G-protein-coupled receptors (GPCRs) (Buck and Axel, 1991; Ngai et al., 1993). Odorants bind to GPCRs leading to the activation of G-proteins, which, in turn, activate adenylate cyclase III. Adenylate cyclase activation triggers the production of cyclic adenosine monophosphate (cAMP) (Breer et al., 1990), which binds to cyclic nucleotide-gated (CNG) channels promoting their opening leading to cation inflow, mainly calcium (Ca^{2+}) and sodium (Na^{+}) ions, depolarizing the membrane potential, eventually generating action potentials (Olivares and Schmachtenberg, 2019). However, there is evidence that, in fish, the phospholipase C/inositol trisphosphate (PLC/IP_3) pathway is involved (Bazáes et al., 2013; Velez et al., 2013) and, possibly, other transduction pathways. Thus, olfactory transduction may depend on external ions for signalling; indeed, olfactory receptor neurons in the olfactory epithelium are in direct contact with the surrounding water and are, therefore, exposed to changes in water chemistry.

Salinity levels constrain the habitable environment of all aquatic organisms; the maintenance of constant plasma ion concentration is crucial to vertebrates. Calcium and sodium, for instance, are involved in a multitude of vital physiological processes such as muscular contraction, action potential generation and cellular signalling. Accumulating evidence suggests that teleosts have olfactory sensitivity to changes in environmental [Ca^{2+}] and [Na^{+}] (Bodznick, 1978; Dew and Pyle, 2014; Herrera et al., 2021; Hubbard et al., 2000, 2002; Nearing et al., 2002; Velez et al., 2009b). Olfactory sensitivity to changes in environmental ions has been described in both freshwater and marine fish; it was recently shown that zebrafish larvae use olfactory detection of Na^{+} and Cl^{-} to avoid water with elevated salinity (Herrera et al., 2021). Goldfish (*Carassius auratus*) and freshwater-reared sockeye salmon (*Oncorhynchus nerka*) have olfactory sensitivity to increases in environmental [Ca^{2+}] and [Na^{+}] (Bodznick, 1978; Hubbard and Canário, 2007; Hubbard et al., 2002); whereas, sole (*Solea senegalensis*), a marine fish that can survive in brackish water, but not freshwater, is sensitive to decreases in [Ca^{2+}] and increases

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in [Na^+] (Velez et al., 2009b). Although olfactory sensitivity to environmental ions depends on species ecology, little is known about how the olfactory system adjusts to waters of such different ionic composition or whether this affects its function and ability to discriminate odorants.

The European seabass, *Dicentrarchus labrax* (hereafter 'seabass'), is primarily marine but uses estuaries as feeding grounds and migrates between seawater and brackish water. Some show long incursions upstream into 100% freshwater (Pickett and Pawson, 1994; Potts, 1995); the reasons for these incursions are not known, but they are facilitated by their capacity to transition between hypo-osmoregulatory and hyper-osmoregulatory modes (Cao et al., 2022; Islam et al., 2021; Jensen et al., 1998; Roche et al., 1989; Varsamos et al., 2001). As with many other fish species, seabass have olfactory sensitivity to amino acids, and disruption of olfactory sensitivity by environmental factors, such as ocean acidification, can cause behavioural alterations (Porteus et al., 2018). Whether movements between different haline environments impact the olfactory system is still to be tested. The main objective of this study was, therefore, to compare olfactory sensitivity in seabass adapted to either seawater (35‰; SW) or brackish water (5‰; BW) to environmental [Na^+] and [Ca^{2+}] and to a range of known odorants conveyed in either freshwater or seawater.

MATERIALS AND METHODS

Fish maintenance

Juvenile seabass, *Dicentrarchus labrax* (Linnaeus 1758), were obtained from a commercial supplier (Mariscos de Esteros SA, Spain) and transported and acclimated indoors at Ramalhete marine station (Universidade do Algarve/CCMAR, Faro, Portugal), where they were kept for 1 year in open flow 1000 l fibreglass tanks supplied with running seawater pumped from the sea, under natural temperature and salinity conditions (after filtration and UV treatment). They were exposed to a simulated natural photoperiod and fed twice a day (2.5% w/w) with a commercial diet (Sparos, Olhão, Portugal). The behaviour and health of all animals were monitored daily and no evidence of infection was observed during the experiments. All procedures followed Portuguese national legislation and EU directives (DL 113/2013 and 2010/63/EU). Protocols were approved by the Ethical Committee ORBEA of CCMAR-University of Algarve and performed under a 'group-2' licence (FELASA C) by the Veterinary General Directorate, Ministry of Agriculture.

Experimental conditions and water chemistry

For adaptation to low salinity, 48 fish (549.3±59.5 g; 34.6±1.4 cm) were randomly distributed into four 500 l tanks (n=12) in an open circuit system kept at natural temperature (19.2±0.2°C for 35‰; 18.0°C±0.2 for 5‰) and a simulated natural photoperiod. Two tanks were kept in control seawater (SW) conditions (35‰) while fish in the other two were gradually acclimated to low salinity (5‰) over 18 days by steadily increasing the inflow/proportion of well brackish water (BW, ∼0.7‰) in the pre-tank mixture (SW:BW).

The brackish flow was increased every 3 days to maintain a step-wise decrease in salinity (5‰/3 days) until the final salinity (∼5‰) was reached. The remaining two tanks received seawater only, at an identical flow (∼3 full turnovers per day). All fish were then left undisturbed, except for normal husbandry, for 3 weeks until terminal sampling or use in electrophysiological experiments.

Water chemistry parameters are shown in Table 1. The methods used were the same as for plasma (see below). The well water used to decrease salinity is slightly brackish and rich in ion content, which conditioned the ionic content of the 5‰ seawater, which is thus higher than expected if distilled water had been used.

Table 1. Seawater chemistry parameters in 35‰ and 5‰ seawater tanks

Parameter	35‰ sea water	5‰ sea water
Osmolarity (mOsm kg^{-1})	1110	206
Na^+ (mmol l^{-1})	550	107
Cl^- (mmol l^{-1})	524	92
Ca^{2+} (mmol l^{-1})	8.9	3.6
K^+ (mmol l^{-1})	13.1	2.3
Mg^{2+} (mmol l^{-1})	51.6	2.9

Sampling and analytical procedures

A month after the acclimation described above started, 10 fish per condition (5 per tank), were euthanized for tissue and fluid collection. Briefly, fish were swiftly collected from their tanks and placed in water with an overdose of 2-phenoxy-ethanol (1:250 v/v). Blood was obtained from the caudal vasculature using a 1 ml syringe fitted with a 21 G heparinized needle and placed on ice. Animals were weighed and measured, and euthanized by sectioning of the cervical column. Blood was centrifuged (5 min at 9000 ***g***) and plasma collected into a clean vial and stored at −80°C until further analysis. Gill filaments were collected, placed in ice-cold sucrose–EDTA–imidazole (SEI) buffer, then snap frozen in liquid nitrogen for Na^+/K^+-ATPase (NKA) activity determination. Olfactory rosettes were dissected and placed in 4% paraformaldehyde (Sigma-Aldrich, Lisbon, Portugal) for histological studies. Dorsal muscle was collected into pre-weighed microtubes and frozen at −20°C for later determination of hydration levels.

Osmoregulatory parameters

Plasma osmolality was measured with a vapour pressure osmometer (VAPRO 5520, Wescor, Logan, UT, USA). The plasma ions calcium, magnesium and chloride, and the metabolites lactate and glucose were measured using commercial complexation and enzymatic colorimetric reactions (Spinreact kits ref. 1001060, 1001285, 1001360, and 1001330 and 1001191, respectively; St Esteve de Bas, Girona, Spain) adapted for 96-well microplates, and optical density (OD) was determined in a microplate reader (MultiScan Go, ThermoFisher Scientific, Tokyo, Japan). Plasma sodium and potassium levels were measured using a flame photometer (BWB XP, BWB Technologies, Newbury, UK). Haematocrit was measured using conventional haematocrit capillary tubes (Hirschmann: www.hirschmann-laborgeraete.de/en/artikelgruppe/91001). pH was measured using a micro-pH probe (Orion™ PerpHecT™ ROSS™ Combination pH Micro Electrode, ThermoFisher Scientific).

NKA activity was measured using the method described by McCormick (1993) with a few modifications. Briefly, gill tissue was thawed on ice and homogenized in 125 ml SEI buffer with 0.1% deoxycholic acid and centrifuged at 2000 ***g*** for 30 s. Quadruplicate 10 µl homogenate samples were added to a 200 µl assay mixture in the presence or absence of 0.5 mmol l^{-1} ouabain (O3125, Sigma-Aldrich) in 96-well microplates at 25°C and absorbance was read at 340 nm for 10 min in a kinetic protocol with intermittent shaking using a microplate reader (MultiScan Go, ThermoFisher Scientific). ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as mmol ADP mg^{-1} protein h^{-1}, which was reduced by the presence of ouabain. The difference in slopes of the curves with or without the pump blocker was used for subsequent calculations of specific

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activity. Sample protein concentration was assayed in duplicate in microplates, using the modified Bradford method with the Quick Start™ Bradford Protein Assay (500-0202, Bio-Rad Laboratories, Lda, Amadora, Portugal) and OD was measured at 595 nm in the above-mentioned microplate reader.

Dorsal white muscle fragments in pre-weighed microtubes were defrosted, weighed with and without the tube, and oven-dried for 96 h at 100°C. Dry muscle was weighed again and the difference was deemed as the mass of evaporated water. Percentage hydration was calculated as the ratio between dry and wet muscle mass (100−dry/wet×100).

Histology

To characterize the general morphology of the olfactory rosettes in seawater-adapted (n=3) and in brackish water-adapted (n=3) seabass, tissues were fixed in 4% PFA, embedded in paraffin and cut into 5 µm sections for H&E staining (Fischer et al., 2008). Stained sections were observed under a microscope (Leica DM2000) coupled to a digital camera (Leica DFC480; IM50-software) linked to a computer, for digital image analysis. The software ImageJ was used to determine the number of mucous cells in the non-sensory epithelium as well as the ratio between the non-sensory epithelium versus sensory epithelium; this was obtained by dividing the length of the apical non-sensory epithelium by the total length of the olfactory lamella (from the top to the central raphe), as previously described (Velez et al., 2019). Images were analysed using the software Fiji ImageJ.

Electrophysiology

Seabass were anaesthetized in aerated natural seawater or 5‰ seawater, as appropriate, containing 300 mg l^{-1} MS222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma-Aldrich) until the response to a tail pinch had stopped; an intramuscular injection of the neuromuscular blocker gallamine triethiodide (Sigma-Aldrich; 10 mg kg^{-1} in 0.9% NaCl) was then given. Fish were then placed in a padded V-support and the gills flushed with aerated seawater containing 150 mg l^{-1} MS222 buffered with 300 mg l^{-1} $NaHCO_3$.

The olfactory rosette was exposed by cutting the skin and connective tissue overlying the nasal cavity. The nostril was constantly irrigated with filtered 35‰ seawater or freshwater (de-chlorinated, charcoal-filtered tap water) without anaesthetic under gravity (flow rate: 10 ml min^{-1}) via a glass tube. Test solutions were delivered to the tube irrigating the nasal cavity via a three-way solenoid valve for 4 s. The olfactory nerves were exposed by removal of the skin, connective tissue and overlying bone between the eyes (Hubbard and Velez, 2020; Hubbard et al., 2000). Charcoal-filtered 35‰ seawater or freshwater was used to make up the odorant solutions and to irrigate the olfactory rosette during experiments. Amino acid solutions were prepared from 20 mmol l^{-1} frozen aliquots; all stimuli were diluted in charcoal-filtered seawater or freshwater (as appropriate) immediately prior to use. The order in which odorants were given was randomized, but each odorant was always given from the lowest to the highest concentration.

Tungsten micro-electrodes (0.1 MΩ, World Precision Instruments, www.wpi-europe.com/index.aspx) were placed in the olfactory nerve in a position that gave maximal response to 1 mmol l^{-1} L-cysteine (Hubbard et al., 2000). Fish were earthed via a copper wire inserted in the flank. The raw signal was amplified (×20,000; AC pre-amplifier, Neurolog NL104, Digitimer Ltd, Welwyn Garden City, UK; www.digitimer.com/), filtered (high pass: 200 Hz, low pass: 3000 Hz; Neurolog NL125, Digitimer Ltd) and integrated (time constant 1 s; Neurolog NL703, Digitimer Ltd). Raw and integrated signals were digitized (Digidata 1440A, Molecular Devices, www.moleculardevices.com/) and recorded on a PC running AxoScope software (version 10.6, Molecular Devices). All integrated response amplitudes were blank subtracted and normalized to the amplitude of the integrated response to 1 mmol l^{-1} L-cysteine (in 35‰ seawater or freshwater as appropriate).

Artificial seawater (ASW) and artificial freshwater (AFW) with and without calcium, with and without sodium, and without both ions were prepared with the composition shown in Table 2. In solutions in which one or both cations were absent, choline chloride was added to maintain osmolality. Each water mixture was used to generate serial dilutions to obtain [Ca^{2+}] ranges (10–0 mmol l^{-1}) or [Na^{+}] ranges (0–460 mmol l^{-1}). When testing the olfactory sensitivity in the absence of external Ca^{2+}, the background water perfusing the olfactory epithelium was Ca^{2+}-free ASW or AFW (as appropriate). When assessing the olfactory sensitivity in the absence of external Na^{+} and/or Ca^{2+}, the background water superfusing the olfactory epithelium also lacked those ions. Experiments with ASW were carried out in fish adapted to 35‰ seawater, and experiments with AFW were carried out in fish adapted to 5‰ seawater.

The different concentrations (10^{-7} to 10^{-3} mol l^{-1}) of the four amino acids (L-alanine, L-glutamic acid, L-arginine and L-leucine) were prepared with the same water perfusing the olfactory epithelium (ASW or AFW as appropriate). To assess the effect of the absence of either Ca^{2+} or Na^{+} on the olfactory responses to amino acids, solutions were made up in Ca^{2+}-free or Na^{+}-free water (ASW or AFW as appropriate); the same water was used as background.

Data and statistical analysis

Differences in the water content of the muscle, activity of the enzyme Na^{+}/K^{+}-ATPase in the gills, plasma osmolarity, and lactate, glucose and ion concentration of control and treated fish were assessed using Student's t-test. Statistically significant differences between the number of goblet cells and the ratio of non-sensory to sensory epithelium in seawater- and brackish water-adapted animals

Table 2. Composition of artificial seawater (ASW) and artificial freshwater (AFW) with and without calcium, with and without sodium, and with and without choline

ASW/AFW	[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
Ca^{2+} and Na^{+}-free ASW	–	10	–	25	25	480
AFW	0.1	–	0.02	–	–	–
Ca^{2+}-free AFW	0.1	–	–	–	–	0.04
Na^{+}-free AFW	–	–	0.02	–	–	0.1
Ca^{2+} and Na^{+}-free AFW	–	–	–	–	–	0.12

All concentrations are 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.

was assessed using Student's *t*-test. For multiple *t*-test analysis, the adjusted *P*-value (*P*′) was calculated with the Bonferroni procedure (Menyhart et al., 2021).

Responses to odorants (when applicable) were described by a three-parameter Hill curve as previously described (Hubbard et al., 2000). The half-maximal effective concentration (EC_{50}) and peak amplitude (I_{max}) were calculated for each fish. These data were then compared using Student's *t*-test. When responses could not fit in a non-linear regression or linear regression, comparisons were done by two-way repeated measures ANOVA with salinity of the water and concentration of the odorants as the two factors and Bonferroni test for multiple comparisons. All analyses were done using SigmaPlot 14; a significance cut-off was set at $P<0.05$ and data are presented as means±s.e.m., unless otherwise stated.

RESULTS

Osmoregulatory parameters

There were no statistical differences in blood pH ($P'=3.48$), haematocrit ($P'=5.64$), plasma osmolarity ($P'=3.96$), glucose ($P'=6.48$), lactate ($P'=1.44$), calcium ($P'=7.8$), sodium ($P'=1.07$), potassium ($P'=11.52$), chloride ($P'=0.59$) and magnesium ($P'=0.24$) between seawater- and brackish water-adapted fish, nor any significant differences between muscle water content ($P'=3.72$) and the activities of the Na^+/K^+-ATPases in the gills ($P'=6.72$) of fish adapted to different salinities.

Histology

In seabass, the olfactory epithelium is located on multilamellar mucosal folds that form a flower-like arrangement – the olfactory rosette. The olfactory lamellae radiate from a central raphe (CR; Fig. 1). There were no marked differences in the gross anatomy of the olfactory organ between fish kept at different salinities. Histologically, each lamella has two layers of epithelium that encloses the central core (CC; Fig. 1). The non-sensory area consists of a stratified epithelium covering the outer margins and the cleft between two adjacent lamellae (NSE; Fig. 1A,B). The sensory area consists of pseudostratified epithelium covering the sides of the lamella (SE; Fig. 1A,B).

There were no significant differences between the ratio of non-sensory and sensory epithelium of seabass kept at different salinities. However, there were significantly ($P<0.001$) more goblet cells per lamella in fish kept in brackish water (23.4±2.4) than in seawater fish (13.9±0.7). Goblets cells were located mainly in non-sensory areas, specifically in the outer margin (Fig. 1C,D) and in the clefts between the lamellae (Fig. 1E,F) close to the central raphe.

Electrophysiology

Given that the same data were used in different analyses, to make the graphs easier to understand, olfactory responses of brackish water-adapted fish are shown as a triangle and responses of seawater-adapted bass are represented by a circle. When the nostril was exposed to, and the odorants delivered in, freshwater, curves are in red; exposure to seawater is in blue.

In seawater-adapted bass, response amplitudes to L-alanine (Fig. 2A), L-glutamic acid (Fig. 2B), L-leucine (Fig. 2C) and L-arginine (Fig. 2D) were lower when the odorants were delivered in freshwater. Except for L-arginine, there were no differences between the EC_{50} values of the amino acids tested in seawater- and brackish water-adapted fish. In bass adapted to brackish water, there were no significant differences between olfactory sensitivity to L-alanine and L-glutamic acid when delivered in seawater or freshwater (Fig. 3A,B); however, brackish water-adapted bass were slightly more sensitive to L-leucine and L-arginine when delivered in seawater than in freshwater. The I_{max} of olfactory sensitivity to L-leucine was significantly higher ($P=0.018$) when the nostril was perfused with seawater than with freshwater (Fig. 3C). For L-arginine, the olfactory response was significantly higher ($P=0.009$) in seawater than in freshwater (Fig. 3D).

Comparison of the olfactory sensitivity of fish adapted to seawater and brackish water when odorants were delivered in seawater showed no significant differences between the EC_{50} for any of the amino acids tested (Fig. 4). However, for L-leucine and L-arginine, the I_{max} was significantly higher ($P=0.035$ and $P=0.006$, respectively) in brackish water-adapted fish than in seawater-adapted fish (Fig. 4C,D).

Comparing the olfactory sensitivity of brackish water- and seawater-adapted fish when odorants were delivered in freshwater, responses to L-alanine, L-glutamic acid, L-leucine and L-arginine were higher in brackish water-adapted than in seawater-adapted fish (Fig. 5). For L-alanine, L-glutamic acid and L-leucine, there were no differences between the EC_{50} values of brackish water-adapted and seawater-adapted fish; however, the I_{max} was significantly higher in brackish water-adapted fish. For L-alanine ($P=0.006$), responses of brackish water-adapted fish had an average I_{max} of 0.9±0.16 and for seawater-adapted bass the I_{max} was 0.5±0.1 (Fig. 5A). For L-glutamic acid ($P=0.029$), the I_{max} was 0.76±0.08 in brackish water-adapted fish and 0.5±0.04 in seawater-adapted fish (Fig. 5B). For L-leucine ($P<0.001$), the olfactory response in brackish water-adapted fish was 0.72±0.05 and in seawater-adapted fish it was 0.35 ±0.06 (Fig. 5C). For L-arginine (Fig. 5D), the EC_{50} was significantly lower ($P=0.001$) in brackish water-adapted fish (1.4×10^{-5} mol l^{-1}) than in seawater-adapted fish (7.2×10^{-5} mol l^{-1}). In addition, the I_{max} for olfactory responses to L-arginine in freshwater was significantly higher ($P<0.001$) in brackish water-adapted fish (0.72±0.05) than in seawater-adapted fish (0.18±0.04).

Seawater-adapted bass were sensitive to decreases, but not increases, in external [Ca^{2+}] (Fig. 6A); in contrast, brackish water-adapted fish were sensitive to increases, but not decreases, in external [Ca^{2+}]. Both, seawater- and brackish water-adapted fish were sensitive to increases in external [Na^+] (Fig. 6B); for the lowest concentration of Na^+ tested, there was no significant difference in the olfactory responses of brackish water- and seawater-adapted fish; however, for all higher [Na^+], the olfactory responses of brackish water-adapted fish to changes in [Na^+] were higher than those of seawater-adapted fish.

The olfactory sensitivity to L-alanine did not depend on the presence of external sodium (Fig. 7). For seawater-adapted fish (Fig. 7A), there were no significant differences between olfactory sensitivity to 10^{-6} and 10^{-4} mol l^{-1} L-alanine in ASW and Na^+-free ASW; however, at 10^{-5} mol l^{-1} L-alanine, the olfactory response was significantly higher ($P=0.027$) in ASW than in Na^+-free ASW. At 10^{-3} mol l^{-1} L-alanine, the olfactory sensitivity of seawater-adapted fish was significantly lower ($P=0.005$) in ASW than in Na^+-free ASW. For brackish water-adapted fish (Fig. 7B), there were no differences between olfactory sensitivity to L-alanine in AFW and Na^+-free AFW. Seawater-adapted fish failed to respond to L-alanine in Ca^{2+}-free ASW, at all concentrations tested (Fig. 7A). Brackish water-adapted fish responded to L-alanine in the absence of external Ca^{2+}; however, there was a significant decrease in response amplitude in Ca^{2+}-free AFW at all concentrations tested (Fig. 7B).

DISCUSSION

The European seabass is a euryhaline fish that commonly uses estuaries as nursery and hunting grounds, being exposed to a large

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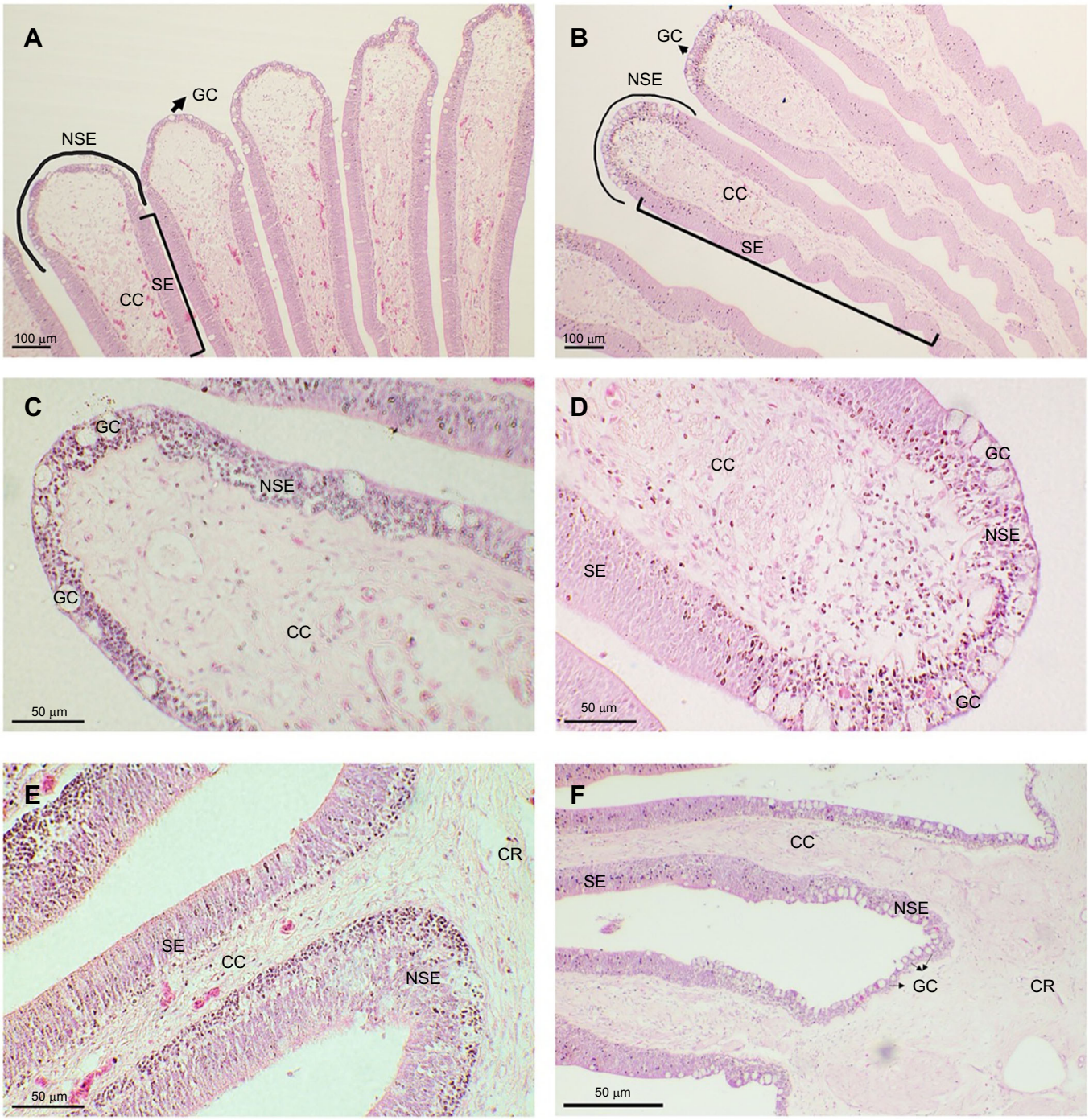

Fig. 1. Morphology of the olfactory rosettes in seawater- and brackish water-acclimated seabass. Representative histological sections of the olfactory rosette of a seawater- (A) and brackish water-acclimated (B) seabass. Outer margin of the olfactory lamellae of a seawater- (C) and brackish water-acclimated (D) seabass. Cleft of lamellae from seawater- (E) and brackish water-acclimated (F) fish. Samples were stained with haematoxylin and eosin. CC, central core; CR, central raphe; NSE, non-sensory epithelium; SE, sensory epithelium, GC, goblet cells (arrows).

variation of environmental salinities, and wild seabass often venture into freshwater, being found several kilometres up-river (Barnabé, 1989; Kelley, 1988; Pickett and Pawson, 1994; Vasconcelos et al., 2010). Like other members of its family, it shows considerable osmoregulatory abilities that allow a smooth transition between such environments (Jensen et al., 1998; Roche et al., 1989), although some intraspecific variability in hyperosmoregulatory capacity has been shown to occur among cultivated stocks as a result of genetic and functional differences (Guinand et al., 2015; Nebel et al., 2005; Thibaut et al., 2019; Tine et al., 2014). Here, we demonstrate that, despite successful longer term osmoregulatory adaptation, other physiological functions, such as olfactory sensitivity, may be impacted by salinity changes in the short term, with possible influences on the fish's ability to find food, detect conspecific signals or use olfactory cues for spatial orientation.

In many complete or partially euryhaline species, acclimation to hypo-osmotic environments has been described as a two-step process (Arjona et al., 2007; Vargas-Chacoff et al., 2020), in which

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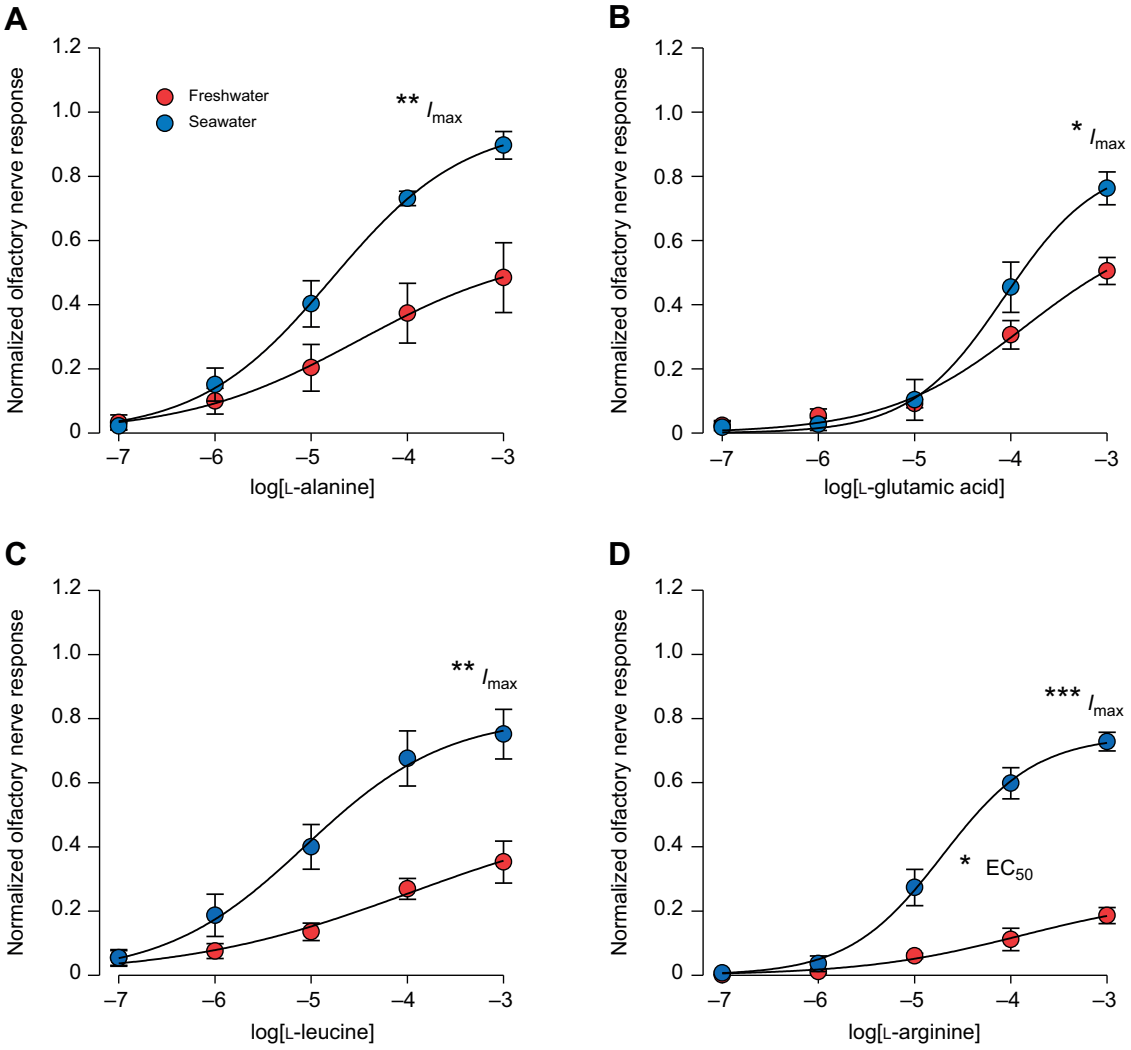

Fig. 2. Olfactory sensitivity of seawater-adapted seabass to amino acids. Responses to different concentrations (mol l^{-1}) of (A) L-alanine, (B) L-glutamic acid, (C) L-leucine and (D) L-arginine when the olfactory epithelium was bathed with freshwater or seawater. Values are shown as means±s.e.m.; *P<0.05, **P<0.01, ***P<0.001 (n=6). I_{max}, maximum amplitude; EC_{50}, half-maximal effective concentration.

major changes in osmoregulatory parameters are often detected within 2–3 days, with relevant modifications in blood osmolality and in the activity of mechanisms such as the branchial Na^+/K^+-ATPase, and followed by a more chronic adjustment period, when these parameters either return to control levels or establish at a new set point. In the present study, adult fish gradually acclimated from 35‰ to 5‰ over 18 days and sampled after a period of 10–12 days at 5‰ showed no differences in plasma osmolality and ionic composition between salinities. This suggests these fish had completely adjusted to the new ionic and osmotic conditions, which was also corroborated by the lack of differences in muscle hydration levels, consistent with an efficient control of water exchange.

Many euryhaline (estuarine, non-migratory) species show a U-shaped relationship between gill Na^+/K^+-ATPase activity and environmental salinity, which is correlated to the dimension of the gradient between internal and external osmolality, usually showing lower activity in salinities with osmolalities closer to the optimal internal milieu of the species (Laiz-Carrión et al., 2005; Ordóñez-Grande et al., 2021; Roche et al., 1989). We have not tested intermediate salinities to evaluate values and responses near the isosmotic point, but our results show similar (P=0.56) branchial Na^+/K^+-ATPase activity between fish at 35‰ and at 5‰. This seems to confirm the U-shaped relationship previously demonstrated in seabass (Jensen et al., 1998) and the acclimation status of brackish water fish.

The current study shows that although, in a euryhaline marine fish, acute exposure to freshwater significantly decreases olfactory sensitivity to amino acids, after adaptation to brackish water, seabass are able to adapt to low salinity, and olfactory sensitivity in freshwater is similar to that in seawater. Seawater-adapted seabass are dependent on external Ca^{2+} for olfactory sensitivity, at least to amino acids; however, brackish water-adapted seabass have normal olfactory sensitivity to amino acids in the absence of environmental Ca^{2+}. This adaptation to low salinity may be, at least in part, due to an increase in the number of mucous cells in the olfactory epithelium producing more – or functionally different – mucus, perhaps maintaining external [Ca^{2+}] in the face of environmental changes. The lack of reliance on external Na^+ ions, in contrast, is indirect evidence for the involvement of the PLC/IP_3 transduction pathway (Kleene, 2008). The olfactory system of seawater-adapted bass (35‰) responds to decreases in environmental [Ca^{2+}], in a similar way to Senegalese sole (*Solea senegalensis*) and seabream (Hubbard et al., 2000; Velez et al., 2009b). However, seabass

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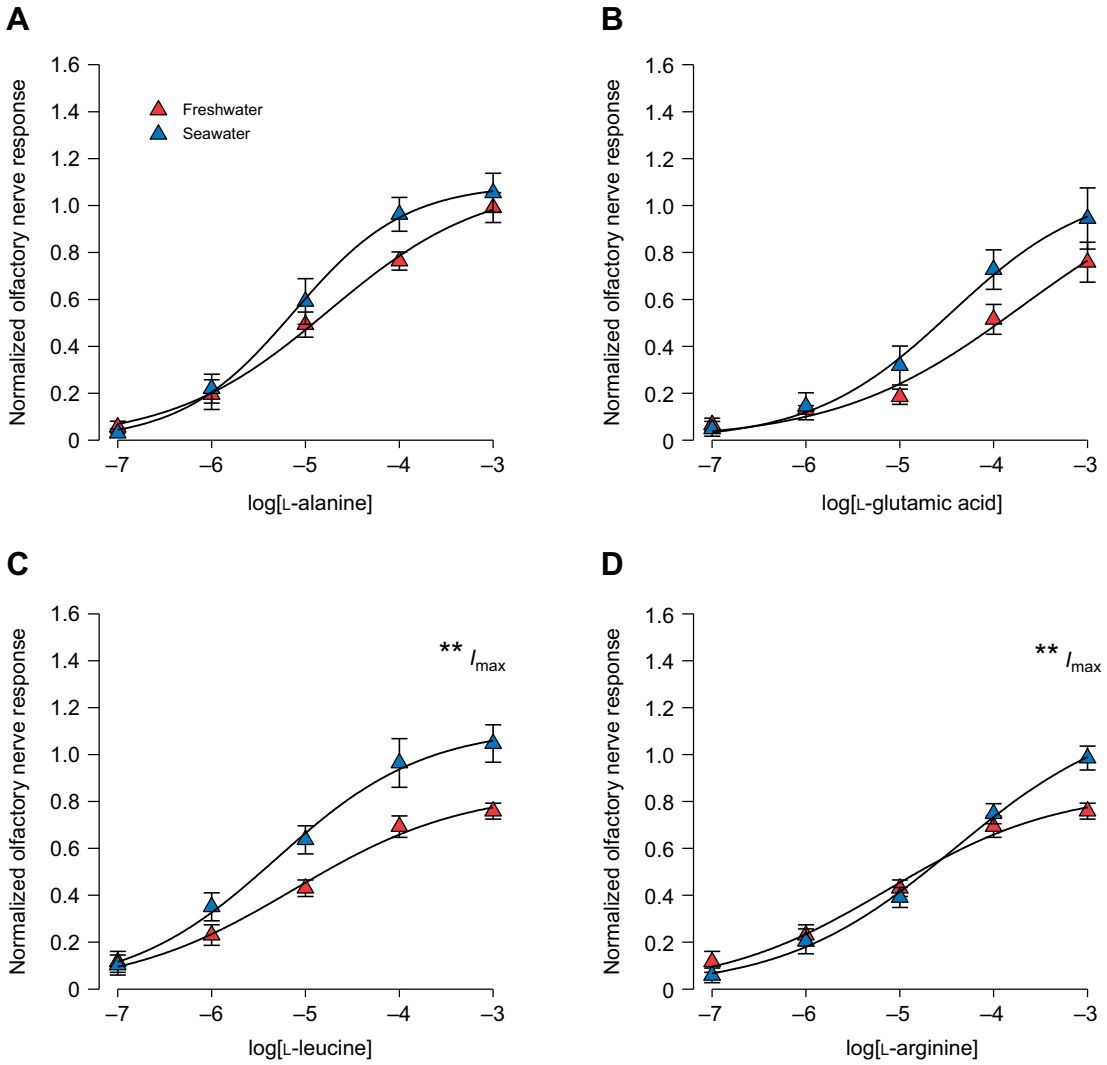

Fig. 3. Olfactory sensitivity of brackish water-adapted seabass to amino acids. Responses to different concentrations (mol l^{-1}) of (A) L-alanine, (B) L-glutamic acid, (C) L-leucine and (D) L-arginine when the olfactory epithelium was bathed with freshwater or seawater. Values are shown as means±s.e.m.; $^{**}P<0.01$ ($n=6$).

adapted to 5‰ seawater are sensitive to increases in environmental [Ca^{2+}]. Similar studies carried out on the Senegalese sole (less tolerant of low salinity than the seabass) showed that both seawater-adapted (35‰) and low salinity-adapted sole (10‰) are sensitive to decreases in environmental [Ca^{2+}], although low salinity-adapted fish are less sensitive than seawater-adapted animals (Velez et al., 2009b). The current study shows that, in the European seabass, a euryhaline species, adaptation to low salinity changes olfactory sensitivity from being sensitive to decreases in [Ca^{2+}] in seawater-adapted fish, to being sensitive to increases in environmental [Ca^{2+}] in brackish water-adapted animals. The olfactory nerve of both seawater- and brackish water-adapted bass responded to increases in environmental [Na^{+}] in a similar way to freshwater-reared salmon, goldfish and sole (Bodznick, 1978; Hubbard et al., 2002; Velez et al., 2009b). The olfactory sensitivity to [Na^{+}] is higher in brackish water- than in seawater-adapted bass, in a similar way that, in the sole, adaptation to low salinity increases olfactory sensitivity to Na^{+} (Velez et al., 2009b).

It was previously suggested that the olfactory sensitivity to Ca^{2+} is mediated by the Ca^{2+}-sensing receptor (Ca^{2+}-SR), similar to that identified in the bovine parathyroid gland (Brown et al., 1993); indeed, this receptor is present in the olfactory epithelia of several fish (Hubbard et al., 2002; Nearing et al., 2002). It is known that the affinity for Ca^{2+} of the mammalian Ca^{2+}-SR is reduced by elevated extracellular [Na^{+}] (Quinn et al., 1998); this is possibly due to 'shielding' of the Ca^{2+} binding site by Na^{+} ions (Loretz, 2008). Indeed, the olfactory sensitivity of goldfish to Ca^{2+} is reduced by increasing environmental Na^{+} (Hubbard and Canário, 2007). However, sole adapted to low salinity are less sensitive to changes in Ca^{2+} (in 10‰ SW) than 35‰ adapted fish (Velez et al., 2009b). The lower sensitivity of 10‰ adapted sole may not be due to lower environmental [Na^{+}] but to lower expression of Ca^{2+}-SR than in 35‰ adapted animals; this may constitute a physiological response of the olfactory system to lower prevailing [Ca^{2+}].

With the current study, we showed that seawater-adapted bass have olfactory sensitivity to decreases in environmental [Ca^{2+}] while brackish water-adapted fish are sensitive to increases in environmental [Ca^{2+}]. Assuming that olfactory responses to Ca^{2+} are mediated by the Ca^{2+}-SR, a possible explanation for sensitivity to decreases in Ca^{2+} may be inhibition of adenylyl cyclase (AC). Indeed, in mammals, activation of Ca^{2+}-SR results in a variety of G-protein-mediated intracellular signals, including activation of

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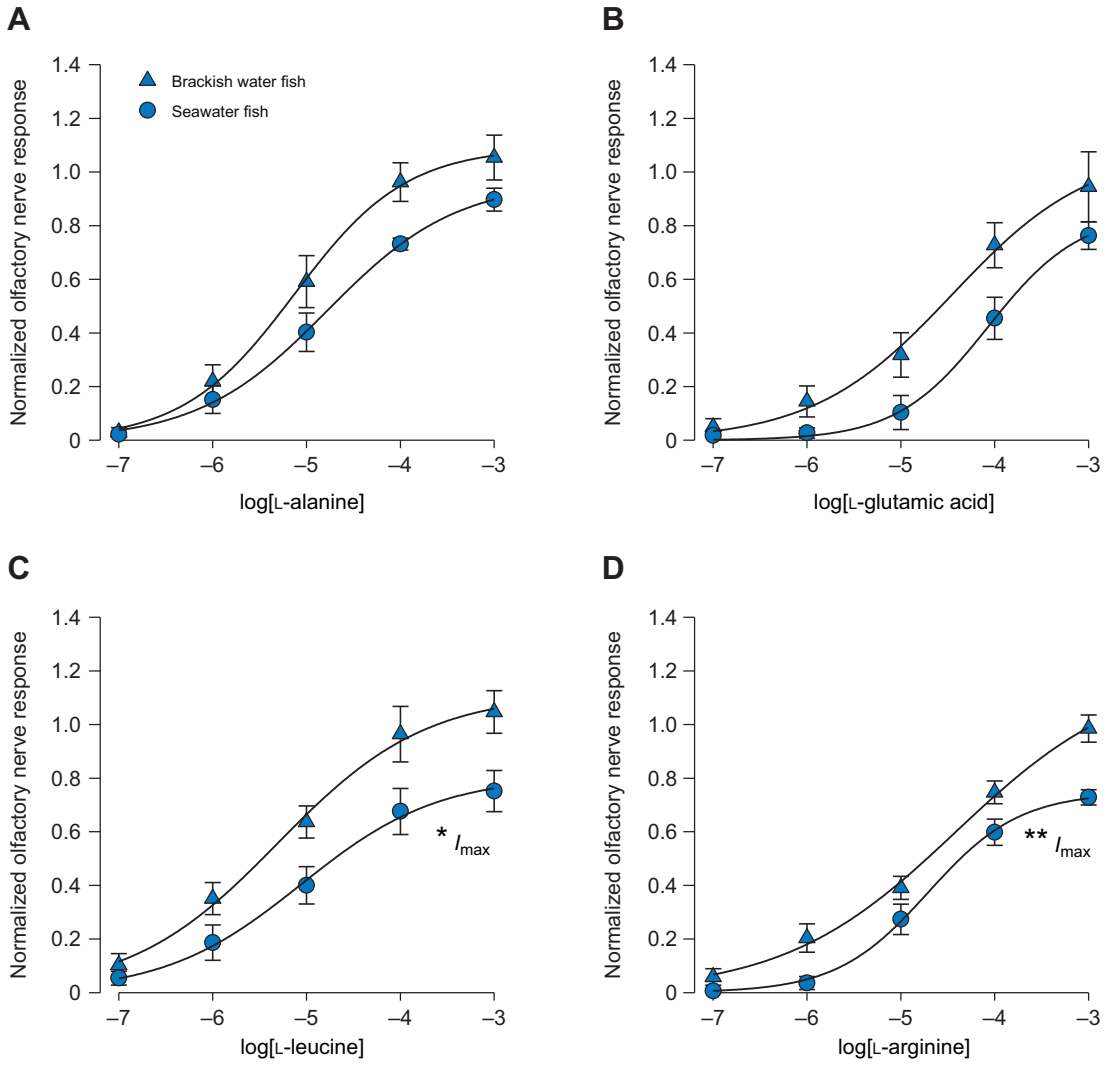

Fig. 4. Olfactory sensitivity of brackish water- and seawater-adapted seabass to amino acids when the olfactory epithelium is bathed with seawater. Responses to different concentrations (mol l^{-1}) of (A) L-alanine, (B) L-glutamic acid, (C) L-leucine and (D) L-arginine. Values are shown as means±s.e.m.; **P*<0.05, ***P*<0.01 (*n*=6).

MAP kinases, PLC, PLA_2 and PI_4K and inhibition of AC (Breitwieser, 2008; Hofer and Brown, 2003). Thus, it may be that, in seawater, Ca^{2+} is continually bound to the receptor 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 neuron depolarization.

Fish, in general, have high olfactory sensitivity to amino acids (Hara, 1994) and seabass is no exception; however, in seawater-adapted fish the olfactory sensitivity to all amino acids tested significantly decreases when the nostril is bathed with freshwater compared with the olfactory sensitivity when the olfactory nostril is bathed with seawater. In brackish water-adapted animals, the olfactory sensitivity in freshwater is similar to that in seawater. This difference in sensitivity between seawater- and brackish water-adapted fish seems to be due to adaptation to low salinity; there was little or no difference between the olfactory sensitivity of brackish water- and seawater-adapted fish when odorants were delivered in seawater. In contrast, the olfactory sensitivity of seawater-adapted fish was significantly lower than that of brackish water-adapted animals when amino acids were delivered in freshwater. Thus, brackish water-adapted animals are able to adapt to low salinity environments. Olfactory sensitivity to L-alanine of both seawater- and brackish water-adapted bass is independent of external Na^{+}; however, in the absence of external Ca^{2+}, seawater-adapted fish are unable to detect L-alanine but brackish water-adapted sea bass can detect L-alanine even in the absence of Ca^{2+}, albeit with reduced sensitivity. At first glance, if seawater-adapted fish were still able to detect alanine in freshwater, the lack of response in the absence of Ca^{2+} seems a contradiction. However, the water used to condition the olfactory epithelium was tap water, which still has some Ca^{2+}, whereas the water used to test the effects of Ca^{2+} and Na^{+} was AFW and ASW, which was prepared with distilled water, and thus had no Ca^{2+}. This suggests that Ca^{2+} is involved in adaptation to low salinity; this may be achieved by changes of the ionic component of the mucous layer overlying the olfactory epithelium, as previously described in mammals (Schild and Restrepo, 1998) or by shifting the transduction pathway to use intracellular rather than extracellular Ca^{2+}; however, this has to be further investigated. Indeed, we found a significant increase in the number of mucous cells in the olfactory epithelium of brackish water-adapted bass, suggesting that mucus is involved in the adaptation of the olfactory system to low salinity; this is in line with studies in seabream that showed that exposure of

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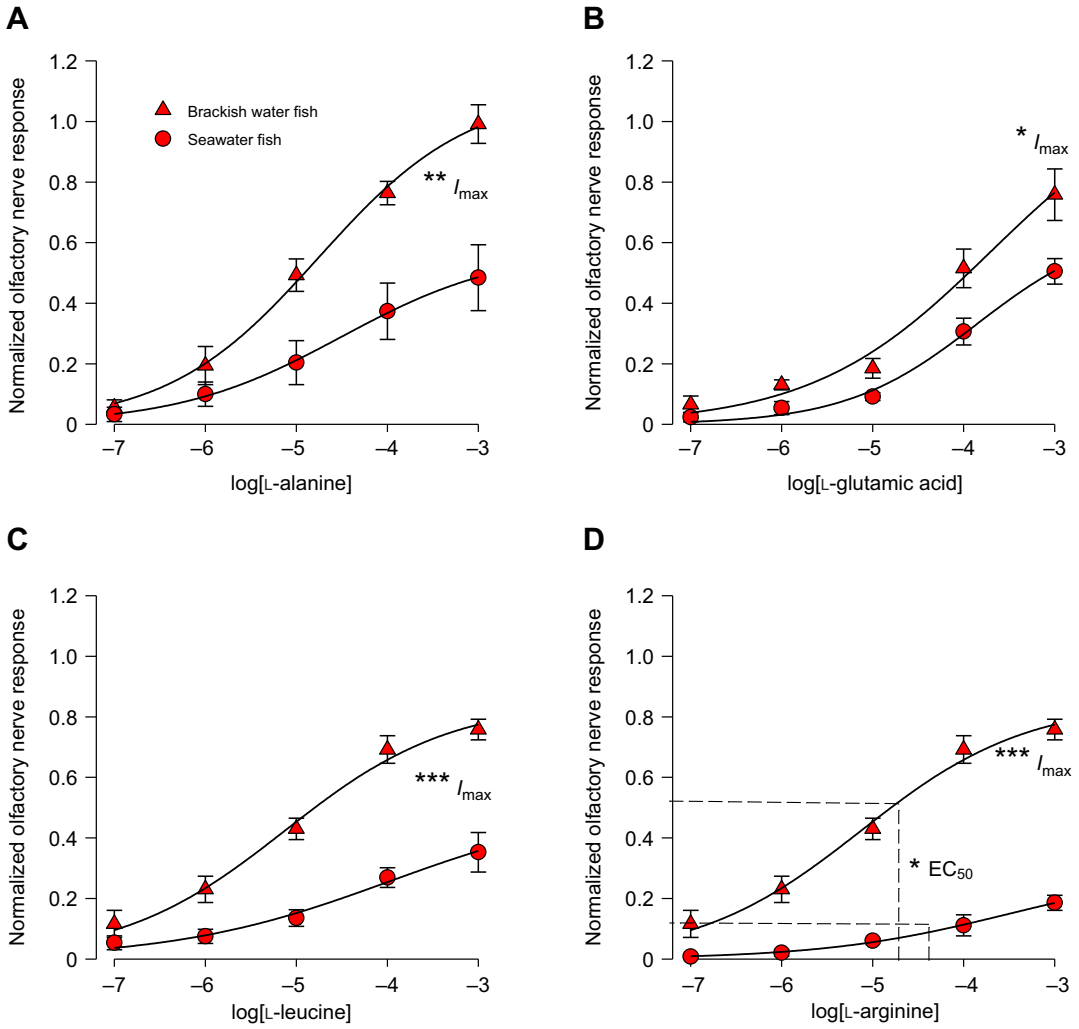
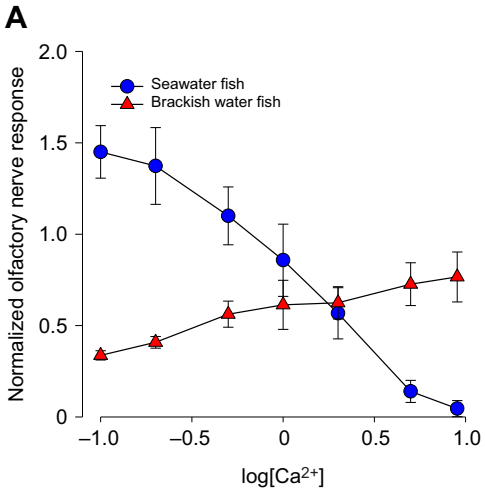


Fig. 5. Olfactory sensitivity of brackish water- and seawater-adapted seabass to amino acids when the olfactory epithelium is bathed with freshwater. Responses to different concentrations (mol l^{-1}) of (A) L-alanine, (B) L-glutamic acid, (C) L-leucine and (D) L-arginine. Values are shown as means ±s.e.m.; *P<0.05, **P<0.01, ***P<0.001 (n=6).

the olfactory epithelium to Ca^{2+}-free ASW causes temporary diminution of the response to L-serine, suggesting some reliance of the transduction mechanism on external Ca^{2+}, which could be overcome (Hubbard et al., 2000). In sole, olfactory sensitivity to amino acids is independent of external Ca^{2+}; however, 35‰ adapted sole were not able to detect amino acids in the absence of Na^+, this is reversed by adaptation to low salinity (Velez et al., 2009a). This may reflect different ion channels (Na^+ selective or Ca^{2+} selective) being



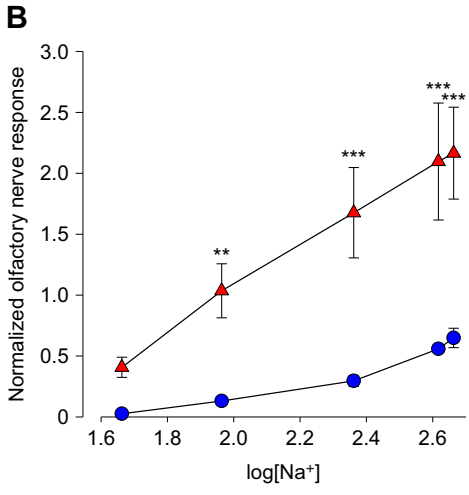


Fig. 6. Olfactory sensitivity of seawater- and brackish water-adapted seabass to changes in external [Ca^{2+}] and [Na^+]. Responses to different concentrations (mmol l^{-1}) of (A) Ca^{2+} and (B) Na^+. Values are shown as means±s.e.m.; **P<0.01, ***P<0.001 (n=6).

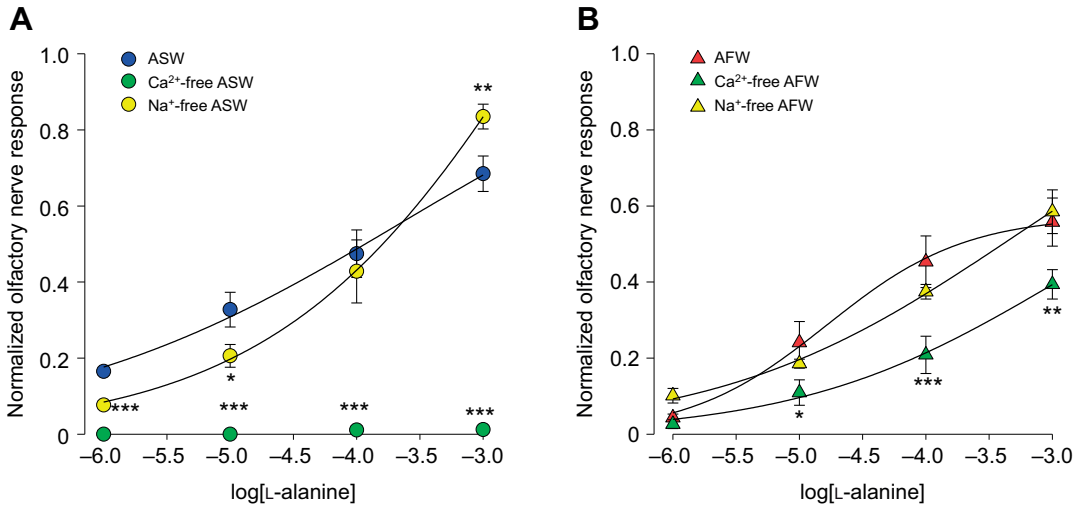


Fig. 7. Effects of external [Na^+] and [Ca^{2+}] on olfactory detection of L-alanine in seawater- and brackish water-adapted seabass. (A) Olfactory detection of L-alanine (mol l^{-1}) in seawater-adapted fish was evaluated in artificial seawater (ASW), Ca^{2+}-free ASW and Na^+-free ASW. (B) Olfactory detection of L-alanine (mol l^{-1}) in brackish water-adapted fish was evaluated in artificial freshwater (AFW), Ca^{2+}-free AFW and Na^+-free AFW. Values are shown as means±s.e.m.; *P<0.05, **P<0.01, ***P<0.001 (n=6).

involved in olfactory signal transduction in different fish species. As far as we are aware, this is the first study on the effects of salinity in terms of the olfactory sensitivity of a euryhaline species living mainly in a marine environment. The current study shows that the olfactory system of seabass is able to adapt to low salinity and, after some time in brackish water, their olfactory sensitivity is as sensitive as in seawater. Considering the vital importance of olfaction in fish survival, this adaptation of the olfactory system to low salinity represents an important feature in the capacity to live in environments of different salinities. However, it is not yet clear how long this adaptation takes; in the short term, bass entering estuarine water of lower salinity would have reduced olfactory sensitivity. Further studies are needed to deepen our understanding of the mechanisms involved in the adaptation of the olfactory system of fishes to different salinities.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: Z.V., P.C.H., P.M.G.; Methodology: Z.V., P.C.H., A.A., R.A.C.; Data curation: Z.V.; Writing - original draft: Z.V.; Writing - review and editing: Z.V., P.C.H., P.M.G.; Supervision: Z.V., P.C.H., P.M.G.

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Data availability
All relevant data can be found within the article and its supplementary information.

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