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RESEARCH ARTICLE

Independent effects of seawater pH and high P_{CO_2} on olfactory sensitivity in fish: possible role of carbonic anhydrase

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ABSTRACT

Ocean acidification may alter olfactory-driven behaviour in fish by direct effects on the peripheral olfactory system; olfactory sensitivity is reduced in CO_2-acidified seawater. The current study tested whether this is due to elevated P_{CO_2} or the consequent reduction in seawater pH and, if the former, the possible involvement of carbonic anhydrase, the enzyme responsible for the hydration of CO_2 and production of carbonic acid. Olfactory sensitivity to amino acids was assessed by extracellular multi-unit recording from the olfactory nerve of the gilthead seabream (*Sparus aurata* L.) in normal seawater (pH ∼8.2), and after acute exposure to acidified seawater (pH ∼7.7) but normal P_{CO_2} (∼340 µatm) or to high P_{CO_2} seawater (∼1400 µatm) at normal pH (∼8.2). Reduced pH in the absence of elevated P_{CO_2} caused a reduction in olfactory sensitivity to L-serine, L-leucine, L-arginine and L-glutamine, but not L-glutamic acid. Increased P_{CO_2} in the absence of changes in pH caused reduced olfactory sensitivity to L-serine, L-leucine and L-arginine, including increases in their threshold of detection, but had no effect on sensitivity to L-glutamine and L-glutamic acid. Inclusion of 1 mmol l^{-1} acetazolamide (a membrane-permeant inhibitor of carbonic anhydrase) in the seawater reversed the inhibition of olfactory sensitivity to L-serine caused by high P_{CO_2}. Ocean acidification may reduce olfactory sensitivity by reductions in seawater pH and intracellular pH (of olfactory receptor neurones); the former by reducing odorant–receptor affinity, and the latter by reducing the efficiency of olfactory transduction. The physiological role of carbonic anhydrase in the olfactory receptor neurones remains to be explored.

KEY WORDS: Ocean acidification, Olfaction, Amino acid, Odorant–receptor affinity, Olfactory receptor, Carbon dioxide

INTRODUCTION

Since the Industrial Revolution, the increase in atmospheric P_{CO_2} due to anthropogenic activity – chiefly the burning of fossil fuels – has had a number of dramatic climatic effects, not least of which is the 'greenhouse effect'. However, oceanic absorption of a proportion of this excess CO_2 has, in turn, caused a decrease in seawater pH from ∼pH 8.2 to a predicted pH 7.7 by the end of the 21st century (Doney et al., 2009; Orr et al., 2005), a process known as ocean acidification. Ocean acidification has been suggested to have a number of sub-lethal, but nevertheless harmful, effects on a wide range of marine organisms (Gunderson et al., 2016). One such effect has been on the olfactory-driven behaviour of fish, wherein fish respond mal-adaptively to olfactory input (Clements and Hunt, 2015; Leduc et al., 2013). For example, larval clownfish (*Amphiprion percula*) normally (at pH 8.15) avoid odours released by predatory rockcod (*Cephalopholis cyanostigma*) and dottyback (*Pseudochromis fuscus*); however, larvae raised in CO_2-acidified seawater (pH 7.80) were attracted to such odours (Dixson et al., 2010). Although the reproducibility of such behavioural studies has been questioned (Clark et al., 2020), the mechanism by which a reduction in seawater pH causes mal-adaptive behavioural responses has been suggested to be a redistribution of extracellular Cl^- and HCO_3^- ions in the cerebrospinal fluid (CSF) which, in turn, causes GABAergic innervation within the CNS to switch from hyperpolarising (inhibitory) to depolarising (excitatory); the '$GABA_A$ receptor theory' (Nilsson et al., 2012).

An alternative or complementary explanation, however, is that the increase in P_{CO_2} and/or decrease in pH has a direct effect on the olfactory system of fish. Electrophysiological studies have shown that exposure to CO_2-acidified seawater causes an immediate and reversible reduction in olfactory sensitivity to some – but not all – odorants for both fish (Porteus et al., 2018; Velez et al., 2019) and crabs (Roggatz et al., 2016). This can be explained – at least in part – by conformational changes in the odorant and/or binding domain of the receptor (as a result of increased protonation), reducing the receptor–ligand binding affinity (Tierney and Atema, 1988; Velez et al., 2019). Nevertheless, the increased protonation cannot, in theory, explain all the observed reduction in olfactory sensitivity. This led us to hypothesise that increased P_{CO_2} may have a direct effect on olfactory sensitivity independent of the reduction in seawater pH. The current study was designed to separate the effects of changes in seawater pH from those of increased P_{CO_2} on olfactory sensitivity in marine fish; that is, could increased CO_2 levels reduce olfactory sensitivity without a concomitant reduction in seawater pH? If so, a possible mechanism is that CO_2 diffuses into the olfactory receptor neurones (ORNs) and causes a reduction of intracellular pH. This could reduce the efficacy or efficiency of the olfactory transduction pathway, thereby reducing olfactory sensitivity. The first hypothesis was tested by comparing olfactory sensitivity of the gilthead seabream (*Sparus aurata*) in normal pH/P_{CO_2} seawater with that in normal pH seawater at high P_{CO_2}, and with that in low pH seawater at normal P_{CO_2}. The second hypothesis was tested by comparing the reduction in olfactory sensitivity due to high P_{CO_2} before and after exposure to acetazolamide, a membrane-permeable inhibitor of carbonic anhydrase that would slow any reduction in intracellular pH due to diffusion of CO_2 into the ORNs.

MATERIALS AND METHODS

Fish maintenance

Animal maintenance and experimentation were carried out in certified experimental facilities and followed Portuguese national legislation (DL 113/2013) under a 'group-1' licence by the

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Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. Gilthead seabream (*Sparus aurata* Linnaeus 1758) (513.3±45 g, 29±1.1 cm; all male, *N*=18) were obtained from a commercial supplier (Maresa – Mariscos de Esteros, SA, Huelva, Spain) and maintained at the experimental station of Ramalhete (Universidade do Algarve, Portugal) in 1000 l tanks with continuously running natural seawater, under natural photoperiod and temperature, and fed daily with commercial pellets (Sparos, Olhão, Portugal).

Water chemistry parameters

During electrophysiological recording, the fish's nostril was irrigated with, and the odorants diluted in, control seawater, high P_{CO_2} seawater or low pH seawater, as appropriate (i.e. only the olfactory system experienced the changes in pH and P_{CO_2}). Control seawater was charcoal-filtered and aerated with ambient air; low pH seawater (with P_{CO_2} equal to that of control seawater) was prepared by decreasing the pH of charcoal-filtered seawater with 1 mol l^{-1} HCl to pH 7.7. High P_{CO_2} water (pH equal to control) was prepared by bubbling CO_2 in charcoal-filtered sea water until pH 7.7 was reached; then, the pH was increased back to pH 8.2 by addition of 1 mol l^{-1} NaOH. As the [Na^+] and [Cl^-] of seawater are high (around 500 mmol l^{-1}), no attempt was made to correct for the addition of HCl or NaOH. Before each experiment, the seawater pH (Orion star A221, Thermo Scientific), alkalinity (DL15 titrator, Mettler Toledo), temperature (Orion star A221, Thermo Scientific) and salinity (WTW cond3310 conductivity meter) were recorded, and the carbonate chemistry parameters were calculated in CO2SYS (Pierrot et al., 2006) using the constants K_1 and K_2 from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and Dickson (1990) for $KHSO_4$. Water chemistry parameters are given in Table 1.

Olfactory nerve recording

Seabream were anaesthetised in aerated natural seawater containing 300 mg l^{-1} MS222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma-Aldrich) until response to tail pinch had stopped; an intramuscular injection of the neuro-muscular blocker gallamine triethiodide (Sigma-Aldrich; 3 mg kg^{-1} in 0.9% NaCl) was then given. Fish were then placed in a padded V-support and the gills irrigated with aerated natural seawater containing 150 mg l^{-1} MS222.

The olfactory rosette was exposed by cutting the skin and connective tissue overlying the nasal cavity. The nostril was constantly irrigated with charcoal-filtered seawater (without anaesthetic) under gravity (flow rate: 6 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 4 s. The olfactory nerve was exposed by removal of the skin, connective tissue and overlying bone (Hubbard et al., 2000). Olfactory nerve activity was recorded using tungsten micro-electrodes (0.1 MΩ, World Precision Instruments) as previously described (Hubbard and Velez, 2020; Hubbard et al., 2000). The electrodes were placed in the olfactory nerve in a position that gave maximal response to 10^{-3} mol l^{-1} L-serine, usually lateral and close to the olfactory bulb. Fish were connected to earth 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), 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 digitised (Digidata 1440A, Molecular Devices, San Jose, CA, USA) and recorded on a PC running AxoScope™ software (version 10.6, Molecular Devices).

All integrated response amplitudes were normalised to the amplitude of the integrated response to 10^{-3} mol l^{-1} L-serine (the 'standard'). Responses to the standard were recorded regularly at the beginning and end of each group of samples (every 3–5 samples) throughout the recording session. Each stimulus was applied for 4 s, with at least 1 min between odorants to allow complete recovery of the receptors.

Stimulus preparation

The effect of acute exposure to high P_{CO_2} and low pH seawater on olfactory sensitivity was tested for a range of amino acids with different side-chain properties (L-serine, L-leucine, L-arginine, L-glutamic acid and L-glutamine); fish have well-established olfactory sensitivity to amino acids (Hara, 1994; Kasumyan, 2004), including the seabream (Hubbard et al., 2003a). Amino acid solutions were prepared from frozen aliquots (10^{-2} mol l^{-1}) and diluted in charcoal-filtered seawater (control, high P_{CO_2} or low pH as appropriate) immediately prior to use. The olfactory epithelium was irrigated with high P_{CO_2} or low pH seawater for 1 min prior to testing the response to odorants. The order in which odorants were tested was varied, but each odorant was tested from lowest to highest concentration. The carbonic anhydrase inhibitor acetazolamide was used to test the involvement of this enzyme in the effects of high P_{CO_2} on olfactory sensitivity to amino acids. A stock solution of 1 mol l^{-1} acetazolamide in dimethyl sulfoxide (DMSO; Sigma-Aldrich) was prepared; immediately before use, a working solution of 1 mmol l^{-1} acetazolamide was prepared by diluting 1 ml of stock solution in 1 l high P_{CO_2} seawater; this was used as background water, and to make the dilutions of L-serine. Two different controls were tested, normal seawater with 0.1% DMSO and high P_{CO_2} seawater with 0.1% DMSO (i.e. both without acetazolamide), in the order: control (normal seawater), 0.1% DMSO in normal seawater, 0.1% DMSO in high P_{CO_2} seawater, then 1 mmol l^{-1} acetazolamine (0.1% DMSO) in high P_{CO_2} seawater. After wash-out of acetazolamide (15 min), a final control concentration–response curve to L-serine in high P_{CO_2} water was run.

Theoretical model for determination of effects of pH on olfactory sensitivity

Some odorant molecules with acidic or basic groups, such as amino acids, can be protonated or not depending on the pH of their surroundings. At a given pH, a mixture of different protonation states may be present. For the current study, we assumed the deprotonated amino acid to be the form binding to the olfactory receptors (Velez et al., 2019). We therefore calculated the concentration of this form (i.e. effective concentration) of different amino acids used in the experiments for the control (pH 8.2) and treatment (pH 7.7)

Table 1. Water parameters

	Low pH experiments		High P_{CO_2} experiments	
	Control	Low pH	Control	High P_{CO_2}
pH_{NBS}	8.17±0.02	7.73±0.01	8.18±0.01	7.68±0.02[2]
Temperature (°C)	24.3±0.9	24.3±0.9	23.6±0.9	23.4±0.9
Salinity	35.2±0.8	35.2±0.8	34.5±0.7	34.5±0.7
Total alkalinity (μmol kg^{-1} seawater)	2802±100	2767±132	2930±162	2980±85
P_{CO_2} (μatm)	338±26	335±29[1]	365±27	1399±88

Data are means±s.e.m. (*N*=6 for each group). [1]Calculated with pH values before the addition of HCl. [2]Values after bubbling with CO_2 but before increasing pH with NaOH to pH 8.2.

conditions based on the concentration used during the bioassay and the group-specific pK_a values for each amino acid obtained from the CRC Handbook of Chemistry and Physics (Lide, 2004). For direct comparison of the change in protonation state and the olfactory response for different concentrations and pH conditions, the respective effective stimulus concentrations were inserted into the linear regression equation/Hill plot obtained from the integrated nerve response at a given pH (see 'Data and statistical analysis', below, for details). The resulting points were plotted in the same figure to show the expected change in olfactory sensitivity caused by stimulus protonation versus the observed change.

Data and statistical analysis

All statistical analyses of electrophysiological results were carried out on normalised data. Differences between olfactory responses at normal pH (8.2) and in acidified water (pH 7.7) or high P_{CO_2} water (P_{CO_2}≈1000 µatm) were analysed by linear regression (L-serine, L-leucine and L-arginine), or fitted to a three-parameter Hill plot (L-glutamic acid and L-glutamine), depending on the standard error of the regression, *S*. Thresholds of detection for L-serine, L-leucine and L-arginine were estimated as the intercept with the *x*-axis using data from individual experiments (Hubbard et al., 2003b), whereas slopes and elevations of pooled data were compared between treatments (Prism 7, GraphPad, La Jolla, CA, USA; www.graphpad.com) using the method for comparing linear regression equations described by Zar (1996, Chapter 17). The slope of each curve indicates how a given increase in odorant concentration evokes a higher amplitude response; this depends on the binding affinity between ligand (odorant in this case) and receptor. Differences between detection thresholds were tested using Student's *t*-test for paired data (log-transformed). Responses to L-glutamine and L-glutamic acid were described by a three-parameter Hill curve as previously described (Hubbard et al., 2000). The half-maximal effective concentration (EC_{50}) and maximum nerve response (I_{max}) were also calculated for each independent experiment for each odorant and each treatment. These data were then compared using Student's *t*-test for paired data (two tailed). A significance level of 0.05 was used throughout.

RESULTS

Effects of odorant pH on olfactory sensitivity

Representative examples of nerve recordings at normal and reduced seawater pH are given in Fig. 1A. The elevation of the concentration–response curves to L-serine (F_{57}=7.63, P<0.01), L-leucine (F_{57}=14.02, P<0.001) and L-arginine (F_{54}=6.45, P<0.05) tested at pH 8.2 was significantly higher than at pH 7.7. However, there were no differences between the slopes of the concentration–response curves at pH 8.2 and 7.7 (Fig. 1B–D). Exposure to low pH water caused significant increases in the thresholds of detection (Fig. 1G).

In contrast, the concentration–response curves of L-glutamine and L-glutamic acid did not fit linear regression well; the best-fitting model was the three-parameter Hill curve (Fig. 1E,F). Olfactory nerve responses of fish exposed to L-glutamic acid at pH 8.2 and 7.7 were statistically equivalent (Fig. 1E). There was no difference (T_5=0.16, P=0.88) between the EC_{50} for L-glutamic acid of fish tested at pH 8.2 (1.61×10^{-4} mol l^{-1}) and pH 7.7 (1.52×10^{-4} mol l^{-1}). In addition, the maximum response (I_{max}) to glutamic acid was equal (T_5=2.403, P=0.07) at pH 8.2 (0.94±0.06) and pH 7.7 (0.80±0.06) (Fig. 1E). Concerning L-glutamine, there was no significant difference (T_5=1.65, P=0.16) between I_{max} at pH 8.2 (1.05±0.05) and pH 7.7 (0.94±0.11); however, the EC_{50} was significantly lower (T_5=2.92, P<0.05) in fish tested at pH 8.2 (4.0×10^{-6} mol l^{-1}) than in fish tested at pH 7.7 (8.25×10^{-6} mol l^{-1}). The olfactory nerve responses at pH 7.7 (at normal P_{CO_2}) were similar to, or slightly lower than, those predicted when calculating the change in protonation status for all amino acids tested, except for the higher concentrations of L-glutamine (Fig. 1F).

Effect of elevated P_{CO_2} on olfactory sensitivity

Representative examples of nerve recordings at normal and increased seawater P_{CO_2} are given in Fig. 2A. The elevation of the concentration–response curves to L-serine (F_{57}=24.12, P<0.001), L-leucine (F_{57}=29.51, P<0.001) and L-arginine (F_{57}=36.89, P<0.001) tested at control P_{CO_2} (~360 µatm) was significantly higher than that in high P_{CO_2} water (~1400 µatm). However, there were no differences between the slope of the concentration–response curves in control and high P_{CO_2} water (Fig. 2B–D). Exposure to high P_{CO_2} water caused significant changes in the threshold of detection for L-serine (T_5=4.45, P<0.01), L-leucine (T_5=2.75, P<0.05) and L-arginine (T_5=2.55, P<0.05). The thresholds of detection for L-serine [$7.99(\pm2.82)\times10^{-8}$ mol l^{-1}], L-leucine [$4.98(\pm2.39)\times10^{-8}$ mol l^{-1}] and L-arginine [$5.98(\pm1.96)\times10^{-8}$ mol l^{-1}] were significantly lower in control seawater than in high P_{CO_2} seawater [$2.46(\pm0.57)\times10^{-7}$ mol l^{-1}, $1.20(\pm0.21)\times10^{-7}$ mol l^{-1} and $1.20(\pm0.21)\times10^{-7}$ mol l^{-1}, respectively; Fig. 2G]. There were no significant differences between the EC_{50} (T_5=1.74, P=0.14) and the I_{max} (T_5=2.32, P=0.07) for olfactory sensitivity to L-glutamic acid (Fig. 2F). In addition, the EC_{50} (T_5=0.11, P=0.92) and the I_{max} (T_5=2.09, P=0.09) for L-glutamine tested in control and high P_{CO_2} water were statistically equivalent (Fig. 2E).

Effect of acetazolamide on olfactory responses in high P_{CO_2} water

There were no significant differences between the slope (F_{66}=0.09, P=0.77) or elevation (F_{67}=0.02, P=0.88) of the concentration–response curves to L-serine in control seawater and control water with DMSO (the vehicle for acetazolamide; data not shown). The slopes of the concentration–response curves to L-serine in control water with DMSO and high P_{CO_2} water with DMSO were statistically identical (F_{66}=2.44; P=0.12); however, the elevation of the curve in control seawater with DMSO was significantly higher (F_{67}=36.83; P<0.001) than that in high P_{CO_2} water with DMSO (Fig. 3). Finally, there were no significant differences between the slope (F_{66}=0.02; P=0.89) or the elevation (F_{67}=2.45; P=0.12) of the concentration–response curves to L-serine in control seawater (with DMSO) and high P_{CO_2} water with acetazolamide (Fig. 3). Thus, sensitivity to L-serine in high P_{CO_2} water in the presence of acetazolamide is statistically equivalent to responses in control water; exposure to 1 mmol l^{-1} acetazolamide reverses the effect of high P_{CO_2} on the olfactory responses to L-serine.

DISCUSSION

The reduction in olfactory sensitivity to amino acids in low pH or high P_{CO_2} seawater in the current study was similar to that previously seen in seabream (Velez et al., 2019) and sea bass (Porteus et al., 2018). This was explained, at least in part, by increased protonation of the odorant molecules and/or their receptors causing a change in charge distribution and 3D conformation and a consequent reduced receptor–odorant affinity (C. S. Porteus, C. C. Roggatz, Z.V., J. D. Hardege and P.C.H., unpublished results). However, the current study shows that, for some odorants at least, olfactory sensitivity in fish may be reduced by increased P_{CO_2} independently of the concomitant decrease in seawater pH. For L-serine, L-leucine and L-arginine, the effect of high P_{CO_2} at normal seawater pH on olfactory sensitivity was as great as, if not greater than, the effect of a reduction in seawater pH

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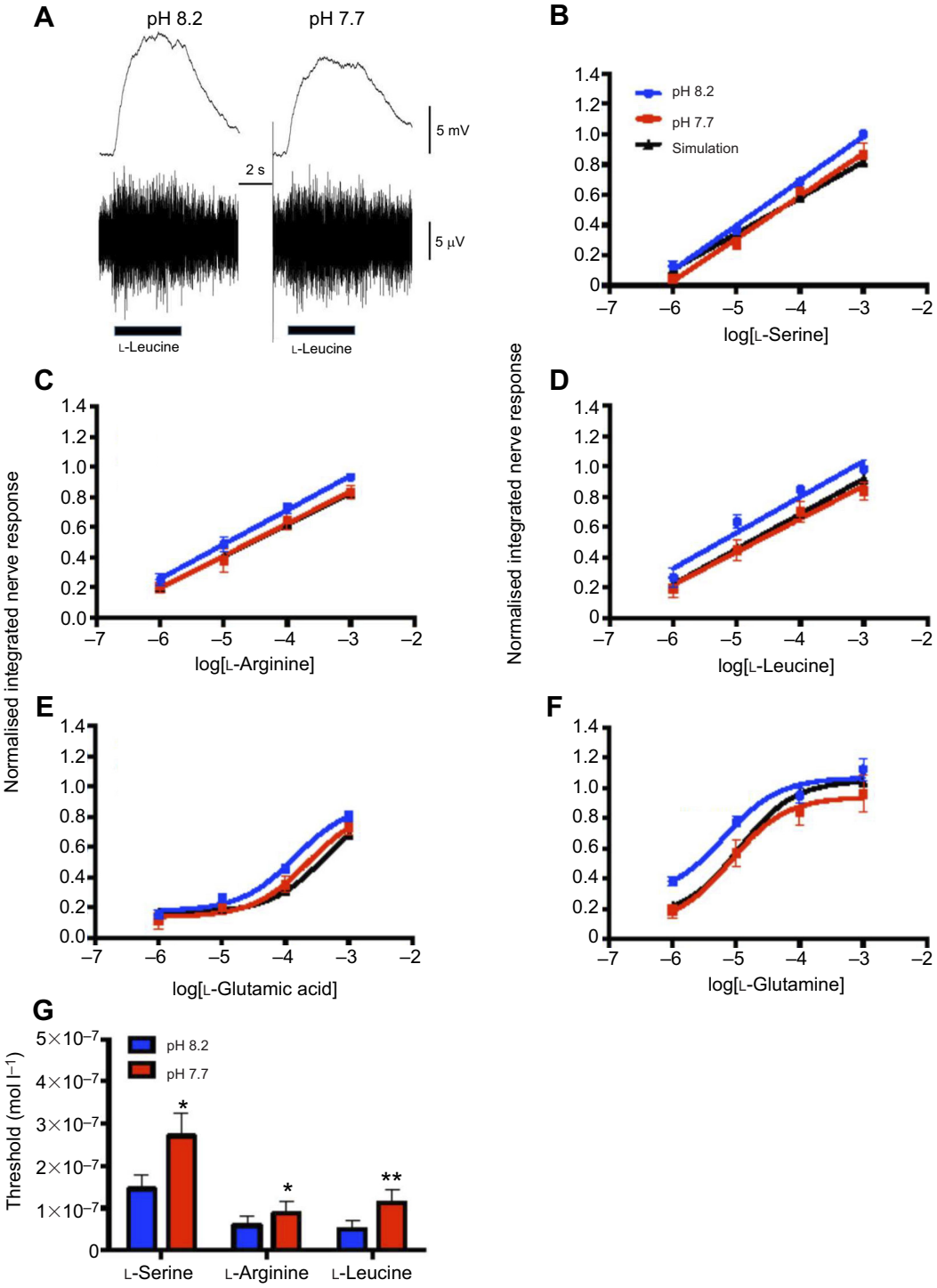

Fig. 1. Effect of decreased seawater pH on olfactory sensitivity in seabream. (A) Example recordings from the olfactory nerve (lower traces) and respective integrated responses (upper traces) to 10^{-4} mol l^{-1} L-leucine (black horizontal bars) in control seawater at pH 8.2 (left traces) and acidified seawater at pH 7.7 (right traces). (B–F) Semi-logarithmic plot of pooled normalised integrated olfactory nerve responses of seabream with the olfactory epithelium exposed to control seawater at pH 8.2 (blue) and seawater at pH 7.7 (red) containing the indicated odorant, and the expected response (black; based on the effective odorant concentration at pH 7.7 and the linear regression equation or three-parameter Hill equation fit) for the following odorants (concentration measured in mol l^{-1}): (B) L-serine, (C) L-arginine, (D) L-leucine, (E) L-glutamic acid and (F) L-glutamine. (G) Effect of exposure to seawater at pH 7.7 on the olfactory thresholds of detection for L-serine, L-leucine and L-arginine. Values are means±s.e.m. (N=6); *P<0.05, **P<0.01.

in the absence of any change in P_{CO_2}. This may be due to diffusion of CO_2 into the ORNs thereby reducing intracellular pH through the production of carbonic acid in a similar way to that of hypercapnia in the mammalian lung (Cortes-Puentes et al., 2019). This is supported by the effect of exposure of the olfactory epithelium to acetazolamide; inhibition of intracellular carbonic anhydrase would slow the fall in intracellular pH when CO_2 diffuses into the ORNs and, in turn, inhibit a consequent reduction in efficiency and/or efficacy of the transduction pathway. Although in tetrapods, increased intracellular [HCO_3^-] activates, rather than inhibits, the catalytic activity of

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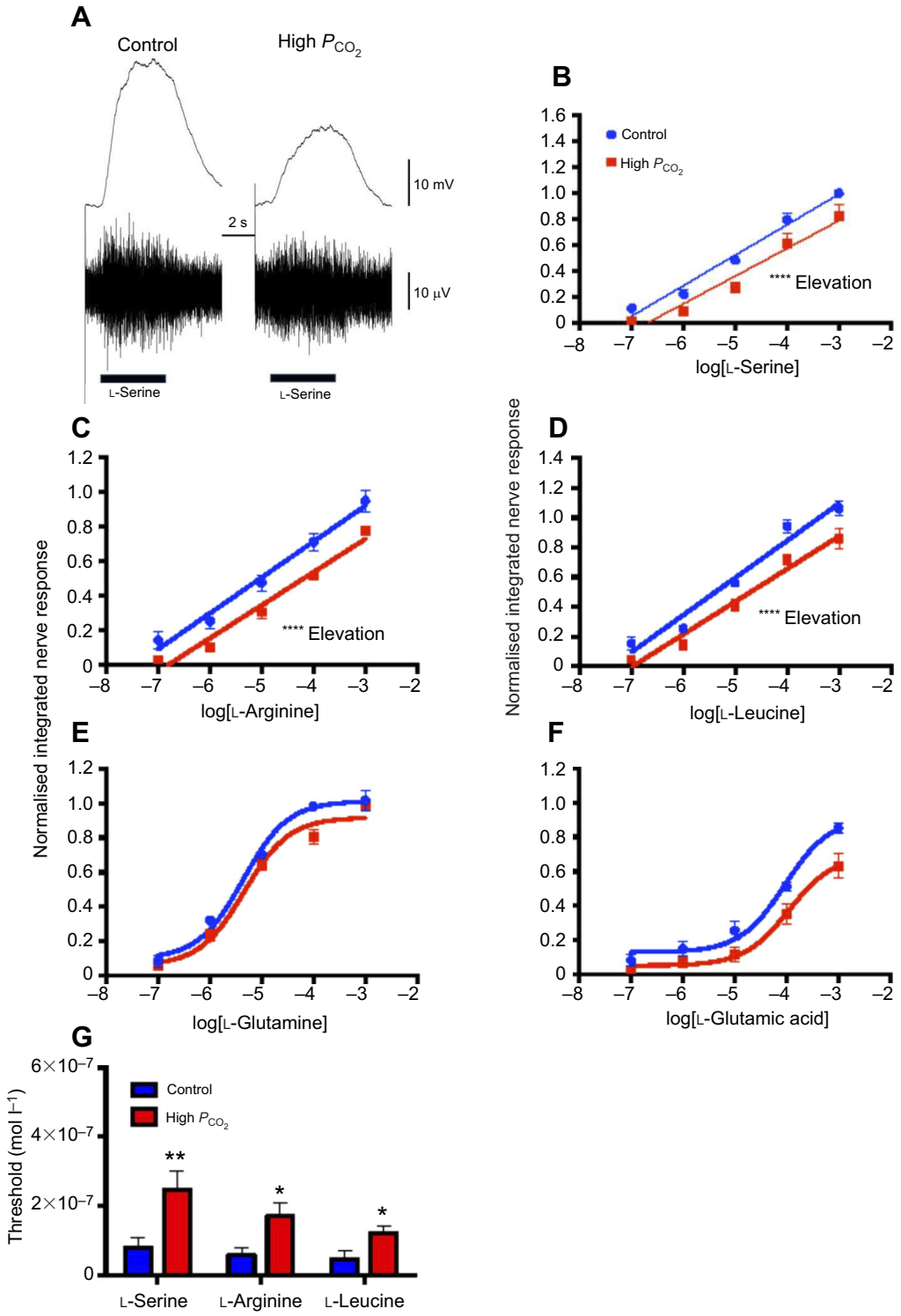

Fig. 2. Effect of increased seawater P_{CO_2} on olfactory sensitivity in seabream. (A) Example recordings from the olfactory nerve (lower traces) and respective integrated responses (upper traces) to 10^{-4} mol l^{-1} L-serine (black horizontal bars) in control seawater P_{CO_2} (left traces) and in seawater with increased P_{CO_2} (right traces). (B–F) Semi-logarithmic plot of pooled normalised integrated olfactory nerve responses of seabream with the olfactory epithelium exposed to control seawater (blue) and high P_{CO_2} water (red) containing the following odorants (concentration measured in mol l^{-1}): (B) L-serine, (C) L-arginine, (D) L-leucine, (E) L-glutamine and (F) L-glutamic acid. (G) Effect of exposure to elevated P_{CO_2} on the olfactory detection threshold for L-serine, L-leucine and L-arginine. Values are means±s.e.m. (N=6); *P<0.05, **P<0.01, ****P<0.001.

adenylate cyclase (Steegborn et al., 2005), blocking this enzyme reduces olfactory transduction efficiency (Chen et al., 2000) and, in fish, high P_{CO_2}/low pH alters expression of key elements in olfactory transduction (Jiahuan et al., 2018). Given that a fall in external pH in the absence of any change in P_{CO_2} would probably lower intracellular [HCO_3^-], and that an increase in P_{CO_2} in the absence of change in external pH would probably increase intracellular [HCO_3^-], the reduction in olfactory sensitivity that occurred in both of these conditions suggests that it is the change in intracellular pH, rather than intracellular [HCO_3^-], that is responsible.

Carbonic anhydrase is a zinc-dependent metalloenzyme that catalyses the hydration of carbon dioxide to produce H^+ and HCO_3^- ions. In fish, it is highly expressed in the gills and, to a lesser extent, in the kidney where it plays a role in acid–base and ionic regulation (Gilmour and Perry, 2009). It is present in a subpopulation of ORNs in tetrapods (Brown et al., 1984; Coates et al., 1998) where it is believed to play a role in the detection of CO_2 and the regulation of ventilation rate (Coates, 2001). It is present in a subpopulation of gill neuroepithelial cells of zebrafish, where it plays a role in the sensitivity of these cells to environmental hypercapnia (Qin et al.,

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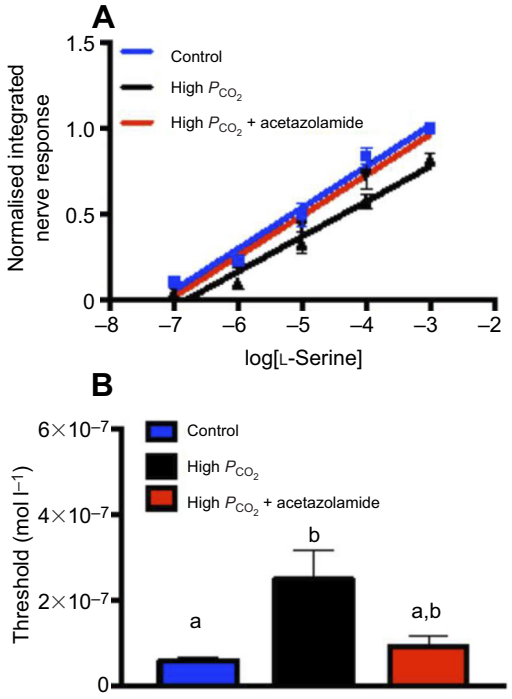

Fig. 3. Effect of acetazolamide on olfactory sensitivity at high P_{CO_2} in seabream. (A) Semi-logarithmic plot of pooled normalised integrated olfactory nerve responses of seabream with the olfactory epithelium exposed to control seawater (+DMSO vehicle; blue; pH 8.17±0.03), high P_{CO_2} seawater (+DMSO; black; pH 8.13±0.04) and high P_{CO_2} water containing 1 mmol l^{-1} acetazolamide in DMSO (red; pH 8.19±0.05) to L-serine. (B) Thresholds of detection for L-serine in control seawater (+DMSO; blue), high P_{CO_2} seawater (+DMSO; red) and 1 mmol l^{-1} acetazolamide in high P_{CO_2} seawater (black). Values are means±s.e.m. (N=6). Bars with different letters are significantly different from each-other.

2010) and the consequent cardiac response (Miller et al., 2014). Its presence and/or function in the olfactory epithelium of fish has, as far as we are aware, not yet been studied. Given that the anatomical link between the olfactory and respiratory systems – present in mammals and other tetrapods – is absent in teleosts, a similar respiratory/cardiovascular role seems unlikely. However, the current study clearly suggests that the presence of carbonic anhydrase in the olfactory system merits further investigation (Milsom, 2012).

That the sensitivity to L-glutamine and L-glutamic acid was not affected by either reduction of seawater pH or increased P_{CO_2} may suggest that a transduction pathway other than adenylate cyclase/cAMP is involved for these odorants. In fish, the transduction pathway for olfactory sensitivity to amino acids, including L-glutamic acid, is thought to be mainly via the phospholipase C pathway (Pang et al., 1994; Velez et al., 2013), although there is evidence for an adenylate cyclase/cAMP pathway (reviewed in Schmachtenberg and Bacigalupo, 2004).

Ocean acidification has been suggested to have detrimental effects on olfactory driven behaviour of fish, and other marine organisms, including food-search, predator avoidance and homing/selection of settling sites (reviewed in Kelley et al., 2018; Leduc et al., 2013; Rivest et al., 2019). In contrast to freshwater fish, in marine fish the mechanism has been suggested to be due to central nervous effects, particularly on $GABA_A$ receptor function (Nilsson et al., 2012). However, one study estimated that a fish in CO_2-acidified seawater needs to be up to 42% closer to an odour source in order to detect it, independently of any central effects (Porteus et al., 2018). This would mean, for example, that predators need to be significantly closer to their prey, and that prey species may allow potential predators to come closer before they are detected. The shift in detection thresholds means that, at environmental concentrations of amino acids (from 10^{-9} to 10^{-7} mol l^{-1}; Williams and Poulet, 1986), CO_2 acidification may make the difference between smelling an amino acid and failing to detect it. Given the central role of amino acids in fish olfaction (Hara, 1994; Kasumyan, 2004), this would have widespread effects on olfactory driven behaviour. Furthermore, that the sensitivity to some odorants is affected more than others means that the qualitative perception, as well as the quantitative detection, of an odour is altered in CO_2-acidified water. This may explain, at least in part, the mal-adaptive behavioural responses to some odours (C. S. Porteus, C. C. Roggatz, Z.V., J. D. Hardege and P.C.H., unpublished results). However, the effects of CO_2-induced acidification on behaviour have been questioned (Clark et al., 2020; see also the reply of Munday et al., 2020). A possible cause of the apparent lack of reproducibility is that the odorants used in such behavioural studies are rarely identified, and are therefore used at unknown concentrations; higher concentrations may overcome the loss of olfactory sensitivity in acidified seawater. Although there are fewer studies addressing the direct effects of CO_2-induced acidification, their results have been consistent.

In conclusion, the current study shows that – in addition to the effect of lowering seawater pH – elevated P_{CO_2} also has a direct effect on the olfactory sensitivity of marine fish. This may be due to lowering of intracellular pH and/or direct effects of increased [HCO_3^-] on the transduction pathway, adenylate cyclase in particular. The physiological significance of carbonic anhydrase in the olfactory epithelium of fish remains to be explored.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.V., P.C.H.; Formal analysis: Z.V., P.C.H.; Investigation: Z.V., R.A.C., W.W., P.C.H.; Writing - original draft: Z.V., P.C.H.; Writing - review & editing: Z.V., R.A.C., W.W., P.C.H.; Supervision: Z.V., R.A.C., P.C.H.; Funding acquisition: Z.V., P.C.H.

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References

Brown, D., Garcia-Segura, L.-M. and Orci, L. (1984). Carbonic anhydrase is present in olfactory receptor cells. *Histochemistry* **80**, 307-309. doi:10.1007/BF00495782

Chen, S., Lane, A. P., Bock, R., Leinders-Zufall, T. and Zufall, F. (2000). Blocking adenylyl cyclase inhibits olfactory generator currents induced by "IP_3-odors". *J. Neurophysiol.* **84**, 575-580. doi:10.1152/jn.2000.84.1.575

Clark, T. D., Raby, G. D., Roche, D. G., Binning, S. A., Speers-Roesch, B., Jutfelt, F. and Sundin, J. (2020). Ocean acidification does not impair the behaviour of coral reef fishes. *Nature* **577**, 370-375. doi:10.1038/s41586-019-1903-y

Clements, J. C. and Hunt, H. L. (2015). Marine animal behaviour in a high CO_2 ocean. *Mar. Ecol. Prog. Ser.* **536**, 259-279. doi:10.3354/meps11426

Coates, E. L. (2001). Olfactory CO_2 chemoreceptors. *Respir. Physiol.* **129**, 219-229. doi:10.1016/S0034-5687(01)00292-4

Coates, E. L., Wells, C. M. Q. and Smith, R. P. (1998). Identification of carbonic anhydrase activity in bullfrog olfactory receptor neurons: histochemical localization and role in CO_2 chemoreception. *J. Comp. Physiol. A* **182**, 163-174. doi:10.1007/s003590050167

Cortes-Puentes, G. A., Westerly, B., Schiavo, D., Wang, S., Stroetz, R., Walters, B., Hubmayr, R. D. and Oeckler, R. A. (2019). Hypercapnia alters alveolar epithelial repair by a pH-dependent and adenylate cyclase-mediated mechanism. *Sci. Rep.* **9**, 349. doi:10.1038/s41598-018-36951-7

Dickson, A. G. (1990). Standard potential of the reaction: AgCl(s) +12H_2(g)=Ag(s)+HCl(aq), and and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. *J. Chem. Thermodyn.* **22**, 113-127. doi:10.1016/0021-9614(90)90074-Z

Dickson, A. G. and Millero, F. J. (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Res. Part A-Oceanographic Res. Pap.* **34**, 1733-1743. doi:10.1016/0198-0149(87)90021-5
Dixson, D. L., Munday, P. L. and Jones, G. P. (2010). Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecol. Lett.* **13**, 68-75. doi:10.1111/j.1461-0248.2009.01400.x
Doney, S. C., Fabry, V. J., Feely, R. A. and Kleypas, J. A. (2009). Ocean acidification: the other CO_2 problem. *Annu. Rev. Mar. Sci.* **1**, 169-192. doi:10.1146/annurev.marine.010908.163834
Gilmour, K. M. and Perry, S. F. (2009). Carbonic anhydrase and acid-base regulation in fish. *J. Exp. Biol.* **212**, 1647-1661. doi:10.1242/jeb.029181
Gunderson, A. R., Armstrong, E. J. and Stillman, J. H. (2016). Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. In *Annual Review of Marine Science*, Vol. 8, (ed. C. A. Carlson and S. J. Giovannoni), p. 357. Palo Alto: Annual Reviews.
Hara, T. J. (1994). The diversity of chemical stimulation in fish olfaction and gustation. *Rev. Fish Biol. Fish.* **4**, 1-35. doi:10.1007/BF00043259
Hubbard, P. and Velez, Z. (2020). Extracellular multi-unit recording from the olfactory nerve of teleosts. *J. Vis. Exp.* doi:10.3791/60962
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., Barata, E. N. and Canário, A. V. M. (2003a). Olfactory sensitivity of the gilthead seabream (*Sparus auratus* L) to conspecific body fluids. *J. Chem. Ecol.* **29**, 2481-2498. doi:10.1023/A:1026357917887
Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2003b). Olfactory sensitivity to catecholamines and their metabolites in the goldfish. *Chem. Senses* **28**, 207-218. doi:10.1093/chemse/28.3.207
Jiahuan, R., Wenhao, S., Xiaofan, G., Wei, S., Shanjie, Z., Maolong, H., Haifeng, W. and Guangxu, L. (2018). Ocean acidification impairs foraging behavior by interfering with olfactory neural signal transduction in black sea bream, *Acanthopagrus schlegelii*. *Front. Physiol.* **9**, 1592. doi:10.3389/fphys.2018.01592
Kasumyan, A. O. (2004). The olfactory system in fish: structure, function, and role in behaviour. *J. Ichthyol.* **44** Suppl. 2, S180-S223.
Kelley, J. L., Chapuis, L., Davies, W. I. L. and Collin, S. P. (2018). Sensory system responses to human-induced environmental change. *Front. Ecol. Evol.* **6**, 95. doi:10.3389/fevo.2018.00095
Leduc, A. O. H. C., Munday, P. L., Brown, G. E. and Ferrari, M. C. O. (2013). Effects of acidification on olfactory-mediated behaviour in freshwater and marine ecosystems: a synthesis. *Philos. Trans. R. Soc. B Biol. Sci.* **368**, 20120447. doi:10.1098/rstb.2012.0447
Lide, D. R. (2004). *CRC Handbook of Chemistry and Physics 85th Edition*. Boca Raton, Florida: CRC Press, p. 2712.
Mehrbach, C., Culberson, C. H., Hawley, J. E. and Pytkowicx, R. M. (1973). Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* **18**, 897-907. doi:10.4319/lo.1973.18.6.0897
Miller, S., Pollack, J., Bradshaw, J., Kumai, Y. and Perry, S. F. (2014). Cardiac responses to hypercapnia in larval zebrafish (*Danio rerio*): the links between CO_2 chemoreception, catecholamines and carbonic anhydrase. *J. Exp. Biol* **217**, 3569-3578. doi:10.1242/jeb.107987
Milsom, W. K. (2012). New insights into gill chemoreception: receptor distribution and roles in water and air breathing fish. *Respir. Physiol. Neurobiol.* **184**, 326-339. doi:10.1016/j.resp.2012.07.013
Munday, P. L., Dixson, D. L., Welch, M. J., Chivers, D. P., Domenici, P., Grosell, M., Heuer, R. M., Jones, G. P., McCormick, M. I., Meekan, M. et al. (2020). Methods matter in repeating ocean acidification studies. *Nature* **586**, E20-E24. doi:10.1038/s41586-020-2803-x
Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sørensen, C., Watson, S.-A., Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nat. Climate Change* **2**, 201-204. doi:10.1038/nclimate1352
Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F. et al. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681-686. doi:10.1038/nature04095
Pang, J., Lo, Y. H., Chandlee, J. M. and Rhoads, D. E. (1994). A subtype of the metabotropic glutamate receptor family in the olfactory system of Atlantic salmon. *FEBS Lett.* **354**, 301-304. doi:10.1016/0014-5793(94)01149-4
Pierrot, D., Lewis, E. and Wallace, D. (2006). MS Excel programme developed for CO2 system calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.
doi:10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a
Porteus, C. S., Hubbard, P. C., Uren Webster, T. M., van Aerle, R., Canário, A. V. M., Santos, E. M. and Wilson, R. W. (2018). Near-future CO_2 levels impair the olfactory system of a marine fish. *Nat. Climate Change* **8**, 737-743. doi:10.1038/s41558-018-0224-8
Qin, Z., Lewis, J. E. and Perry, S. F. (2010). Zebrafish (*Danio rerio*) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO_2. *J. Physiol.* **588**, 861-872. doi:10.1113/jphysiol.2009.184739
Rivest, E. B., Jellison, B., Ng, G., Satterthwaite, E. V., Bradley, H. L., Williams, S. L. and Gaylord, B. (2019). Mechanisms involving sensory pathway steps inform impacts of global climate change on ecological processes. *Front. Mar. Sci.* **6**, 346. doi:10.3389/fmars.2019.00346
Roggatz, C. C., Lorch, M., Hardege, J. D. and Benoit, D. M. (2016). Ocean acidification affects marine chemical communication by changing structure and function of peptide signalling molecules. *Glob. Change Biol.* **22**, 3914-3926. doi:10.1111/gcb.13354
Schmachtenberg, O. and Bacigalupo, J. (2004). Olfactory transduction in ciliated receptor neurons of the Cabinza grunt, *Isacia conceptionis* (teleostei: haemulidae). *Eur. J. Neurosci.* **20**, 3378-3386. doi:10.1111/j.1460-9568.2004.03825.x
Steegborn, C., Litvin, T. N., Levin, L. R., Buck, J. and Wu, H. (2005). Bicarbonate activation of adenylyl cyclase via promotion of catalytic active site closure and metal recruitment. *Nat. Struct. Mol. Biol.* **12**, 32-37. doi:10.1038/nsmb880
Tierney, A. J. and Atema, J. (1988). Amino acid chemoreception: effects of pH on receptors and stimuli. *J. Chem. Ecol.* **14**, 135-141. doi:10.1007/BF01022537
Velez, Z., Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2013). Olfactory transduction pathways in the Senegalese sole *Solea senegalensis*. *J. Fish Biol.* **83**, 501-514. doi:10.1111/jfb.12185
Velez, Z., Roggatz, C. C., Benoit, D. M., Hardege, J. D. and Hubbard, P. C. (2019). Short- and medium-term exposure to ocean acidification reduces olfactory sensitivity in gilthead seabream. *Front. Physiol.* **10**, 731. doi:10.3389/fphys.2019.00731
Williams, R. and Poulet, S. A. (1986). Relationship between the zooplankton, phytoplankton, particulate matter and dissolved free amino acids in the Celtic Sea. 1. Unstratified water conditions. *Mar. Biol.* **90**, 279-284. doi:10.1007/BF00569139
Zar, J. H. (1996). *Biostatistical Analysis*, p. 800. New Jersey: Pearson Higher Education.