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

Zélia Velez, Rita A. Costa, Wenjing Wang and Peter C. Hubbard*

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 rockeck (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 – odours 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-I’ licence by the
Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. Gilthead seabream (*Sparus aurata* Linnaeus 1758) (513±45 g, 29±1.1 cm; all male, *N*=18) were obtained from a commercial supplier (Maresa – Maricoses 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, Ollhão, Portugal).

**Water chemistry parameters**

During electrophysiological recording, the fish’s nostril was irrigated with, and the odorants diluted in, control seawater, high *P*\textsubscript{CO}\textsubscript{2} seawater or low pH seawater, as appropriate (i.e. only the olfactory system experienced the changes in pH and *P*\textsubscript{CO}\textsubscript{2}). Control seawater was charcoal-filtered and aerated with ambient air; low pH seawater (with *P*\textsubscript{CO}\textsubscript{2} equal to that of control seawater) was prepared by decreasing the pH of charcoal-filtered seawater with 1 mol l\textsuperscript{−1} *H*\textsubscript{2}C\textsubscript{O}\textsubscript{3}\textsuperscript{−1} to pH 7.7. High *P*\textsubscript{CO}\textsubscript{2} water (pH equal to control) was prepared by bubbling CO\textsubscript{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\textsuperscript{−1} NaOH. As the [Na\textsuperscript{+}] and [Cl\textsuperscript{−}] of seawater are high (around 500 mmol l\textsuperscript{−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, 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 and the carbonate chemistry parameters were calculated in CO2SYS and salinity (WTW cond3310 conductivity meter) were recorded, and the carbonate chemistry parameters were calculated in CO2SYS (Pierrot et al., 2006) using the constants and the carbonate chemistry parameters were calculated in CO2SYS (Pierrot et al., 2006) using the constants.

**Olfactory nerve recording**

Seabream were anaesthetised in aerated natural seawater containing 300 mg l\textsuperscript{−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\textsuperscript{−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\textsuperscript{−1} MS222.

The olfactory rosette was exposed by cutting the skin and connective tissue overlying the nasal cavity. The nostril was irrigated with, and the odorants diluted in, control seawater, high *P*\textsubscript{CO}\textsubscript{2} seawater or low pH seawater, as appropriate (i.e. only the olfactory system experienced the changes in pH and *P*\textsubscript{CO}\textsubscript{2}). Control seawater was charcoal-filtered and aerated with ambient air; low pH seawater (with *P*\textsubscript{CO}\textsubscript{2} equal to that of control seawater) was prepared by decreasing the pH of charcoal-filtered seawater with 1 mol l\textsuperscript{−1} *H*\textsubscript{2}C\textsubscript{O}\textsubscript{3}\textsuperscript{−1} to pH 7.7. High *P*\textsubscript{CO}\textsubscript{2} water (pH equal to control) was prepared by bubbling CO\textsubscript{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\textsuperscript{−1} NaOH. As the [Na\textsuperscript{+}] and [Cl\textsuperscript{−}] of seawater are high (around 500 mmol l\textsuperscript{−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*\textsubscript{1} and *K*\textsubscript{2} from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and Dickson (1990) for KHSO\textsubscript{4}. Water chemistry parameters are given in Table 1.

<table>
<thead>
<tr>
<th>Water parameters</th>
<th>Low pH experiments</th>
<th>High <em>P</em>\textsubscript{CO}\textsubscript{2} experiments</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>Low pH</td>
<td>Control</td>
</tr>
<tr>
<td>pH\textsubscript{NBS}</td>
<td>8.17±0.02</td>
<td>7.73±0.01</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>24.3±0.9</td>
<td>23.6±0.9</td>
</tr>
<tr>
<td>Salinity</td>
<td>35.2±0.8</td>
<td>34.5±0.7</td>
</tr>
<tr>
<td>Total alkalinity (µmol kg\textsuperscript{−1} seawater)</td>
<td>2802±100</td>
<td>2930±162</td>
</tr>
<tr>
<td><em>P</em>\textsubscript{CO}\textsubscript{2} (µatm)</td>
<td>338±26</td>
<td>365±27</td>
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</table>

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\textsubscript{2} but before increasing pH with NaOH to pH 8.2.

The effect of acute exposure to high *P*\textsubscript{CO}\textsubscript{2} and low pH seawater on olfactory sensitivity was tested for a range of amino acids with different side-chain properties (i-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\textsuperscript{−2} mol l\textsuperscript{−1}) and diluted in charcoal-filtered seawater (control, high *P*\textsubscript{CO}\textsubscript{2} or low pH as appropriate) immediately prior to use. The olfactory epithelium was irrigated with high *P*\textsubscript{CO}\textsubscript{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*\textsubscript{CO}\textsubscript{2} on olfactory sensitivity to amino acids. A stock solution of 1 mol l\textsuperscript{−1} acetazolamide in dimethyl sulfoxide (DMSO; Sigma-Aldrich) was prepared; immediately before use, a working solution of 1 mmol l\textsuperscript{−1} acetazolamide was prepared by diluting 1 ml of stock solution in 1 l high *P*\textsubscript{CO}\textsubscript{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*\textsubscript{CO}\textsubscript{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*\textsubscript{CO}\textsubscript{2} seawater, then 1 mmol l\textsuperscript{−1} acetazolamine (0.1% DMSO) in high *P*\textsubscript{CO}\textsubscript{2} seawater. After wash-out of acetazolamide (15 min), a final control concentration–response curve to l-serine in high *P*\textsubscript{CO}\textsubscript{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)
conditions based on the concentration used during the bioassay and the group-specific pKₐ 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_CO₂ water (P_CO₂∼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 (EC₅₀) 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 (F₅₇=7.63, P<0.01), L-leucine (F₅₇=14.02, P<0.001) and L-arginine (F₅₇=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 (T₅₇=0.16, P=0.88) between the EC₅₀ 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 (T₅₇=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 (T₅₇=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 EC₅₀ was significantly lower (T₅₇=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_CO₂) 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_CO₂ on olfactory sensitivity
Representative examples of nerve recordings at normal and increased seawater P_CO₂ are given in Fig. 2A. The elevation of the concentration–response curves to L-serine (F₅₇=24.12, P<0.001), L-leucine (F₅₇=29.51, P<0.001) and L-arginine (F₅₇=36.89, P<0.001) tested at control P_CO₂ (∼360 µatm) was significantly higher than that in high P_CO₂ water (∼1400 µatm). However, there were no differences between the slope of the concentration–response curves in control and high P_CO₂ water (Fig. 2B–D). Exposure to high P_CO₂ water caused significant changes in the threshold of detection for L-serine (T₅₇=4.45, P<0.01), L-leucine (T₅₇=2.75, P<0.05) and L-arginine (T₅₇=2.55, P<0.05). The thresholds of detection for L-serine [7.99(±2.82)×10⁻⁸ mol l⁻¹], L-leucine [4.98(±2.39)×10⁻⁸ mol l⁻¹] and L-arginine [5.98(±1.96)×10⁻⁸ mol l⁻¹] were significantly lower in control seawater than in high P_CO₂ seawater [2.46(±0.57)×10⁻⁷ mol l⁻¹, 1.20(±0.21)×10⁻⁷ mol l⁻¹ and 1.20(±0.21)×10⁻⁷ mol l⁻¹, respectively; Fig. 2G]. There were no significant differences between the EC₅₀ (T₅₇=1.74, P=0.14) and the I_max (T₅₇=2.32, P=0.07) for olfactory sensitivity to L-glutamic acid (Fig. 2F). In addition, the EC₅₀ (T₅₇=0.11, P=0.92) and the I_max (T₅₇=2.09, P=0.09) for L-glutamine tested in control and high P_CO₂ water were statistically equivalent (Fig. 2E).

Effect of acetazolamide on olfactory responses in high P_CO₂ water
There were no significant differences between the slope (F₆₆=0.09, P=0.77) or elevation (F₆₆=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_CO₂ water with DMSO were statistically identical (F₆₆=2.44; P=0.12); however, the elevation of the curve in control seawater with DMSO was significantly higher (F₆₆=36.83; P<0.001) than that in high P_CO₂ water with DMSO (Fig. 3). Finally, there were no significant differences between the slope (F₆₆=0.02; P=0.89) or the elevation (F₆₆=2.45; P=0.12) of the concentration–response curves to L-serine in control seawater (with DMSO) and high P_CO₂ water with acetazolamide (Fig. 3). Thus, sensitivity to L-serine in high P_CO₂ 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_CO₂ on the olfactory responses to L-serine.

DISCUSSION
The reduction in olfactory sensitivity to amino acids in low pH or high P_CO₂ 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_CO₂ independently of the concomitant decrease in seawater pH. For L-serine, L-leucine and L-arginine, the effect of high P_CO₂ at normal seawater pH on olfactory sensitivity was as great as, if not greater than, the effect of a reduction in seawater pH.
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. 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. ($N=6$); *$P<0.05$, **$P<0.01$. 
adenylate cyclase (Steegborn et al., 2005), blocking this enzyme reduces olfactory transduction efficiency (Chen et al., 2000) and, in fish, high \( P_{\text{CO}_2} \)/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 \( P_{\text{CO}_2} \) would probably lower intracellular \( \text{[HCO}_3^-] \), and that an increase in \( P_{\text{CO}_2} \) in the absence of change in external pH would probably increase intracellular \( \text{[HCO}_3^-] \), the reduction in olfactory sensitivity that occurred in both of these conditions suggests that it is the change in intracellular pH, rather than intracellular \( \text{[HCO}_3^-] \), that is responsible.

Carbonic anhydrase is a zinc-dependent metalloenzyme that catalyses the hydration of carbon dioxide to produce \( H^+ \) and \( \text{HCO}_3^- \) 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 \( \text{CO}_2 \) 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.,...
2010) and the consequent cardiac discharge (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\textsubscript{CO2} may suggest that a transduction pathway other than adenylate cyclase/cAMP 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 Bagcikulo, 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; Rivist 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\textsubscript{A} receptor function (Nilsson et al., 2012). However, one study estimated that a fish in CO\textsubscript{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\textsuperscript{-6} to 10\textsuperscript{-7} mol l\textsuperscript{-1}; Williams and Poulet, 1986), CO\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{CO2} 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\textsubscript{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

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