Near-future carbon dioxide levels impair the olfactory system of a marine fish

Cosima S. Porteus[1*], Peter C. Hubbard[2], Tamsyn M. Uren Webster[3], Ronny van Aerle[4], Adelino V. M. Canário[2], Eduarda M. Santos[1], Rod W. Wilson[1*]

[1]Biosciences, College of Life & Environmental Sciences, University of Exeter, Exeter, Devon, EX4 4QD, UK

[2]Centro de Ciências do Mar, Campus de Gambelas, Universidade do Algarve, Faro, Algarve, 8005-139 Portugal

[3]Biosciences, College of Science, Swansea University, Swansea, Wales, SA2 8PP, UK

[4]Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth, Dorset, DT4 8UB, UK

*Corresponding authors:

Cosima Porteus, email: cosimaporteus@gmail.com

Rod W. Wilson, email: R.W.Wilson@exeter.ac.uk

Survival of marine fishes exposed to elevated near-future CO_2 levels is threatened by their altered responses to sensory cues. Here we demonstrate a novel physiological and molecular mechanism in the olfactory system which helps explain altered behavior under elevated CO_2. We combine electrophysiology and transcriptomics with behavioral experiments to investigate how elevated CO_2 affects the olfactory system of European sea bass (*Dicentrarchus labrax*). Under elevated CO_2 (~1000 μatm) fish must be up to 42% closer to an odor source for detection, compared with current CO_2 levels (~400 μatm), decreasing their chances of detecting food or predators. Compromised olfaction correlated with the suppression in the transcription of genes involved in synaptic strength, cell excitability, and wiring of the olfactory system in response to sustained exposure to elevated CO_2. Our findings complement the previously proposed impairment of γ-aminobutyric acid receptors, suggesting both the olfactory system and central brain function are compromised by elevated CO_2.

Fish rely heavily on their olfaction for finding food[1,2], recognizing conspecifics and predators[1,3,4], for the perception of reproductive status[5] and homing towards suitable habitats[6,7,8,9], including spawning grounds and larval settlement[10]. The predicted end-of-the-century CO_2 levels (800-1000 μatm) cause ocean acidification (elevated H^+, reduced pH) and have been shown to have negative effects on the sensory-related behavior and learning of many fish species studied to-date[3,4,11,12,13] including sharks[14,15]. Moreover, coral reef fishes exposed to elevated CO_2 levels predicted for the year 2100, show a 9-fold increase in mortality, when returned to the wild as compared with fish exposed to current conditions[4]. This suggests the potential for a

major ecologically-relevant impairment of fitness of marine fishes as a consequence of exposure to elevated CO_2.

To explain the effects of elevated CO_2 on fish behavior, changes in brain neurotransmitter function have been proposed as the sole mechanism responsible[11,16,17,18]. To date, there has been no consideration of mechanisms operating outside of the central processing of sensory information. A direct effect of seawater pH or CO_2 on the peripheral olfactory system was dismissed in marine fish[19] without any empirical testing. We have challenged this view and hypothesized that elevated CO_2 would directly affect the olfactory system of fish, given that the olfactory epithelium is in intimate contact with sea water. We propose a novel physiological mechanism by which ocean acidification can alter fish behavior and learning directly via the olfactory system, and used the European sea bass as a model to test this hypothesis.

Results

Firstly, we confirmed that the behavior of European sea bass was affected by elevated CO_2 levels as demonstrated in other fish species. Juvenile sea bass were exposed to current (~430 µatm; control) and predicted end of the century levels of CO_2 (~1000 µatm; elevated CO_2) and their behavioral responses to a likely predator odor[20] (bile from monkfish, *Lophius piscatorius*; dilution 1:1,000,000) was quantified. Our data demonstrated that sea bass exposed to elevated CO_2 reduced their baseline activity (swimming) by up to 40% compared with control fish ($p=0.008$, Fig 1a). This difference was independent of the duration of exposure ($p=0.30$). Furthermore, control sea bass reduced their activity by 50% in the presence of the predator odor, whereas sea bass exposed to elevated CO_2 reduced their activity by only 20-27% ($P=0.009$, Fig. 1b). Both control and elevated CO_2 exposed fish

displayed freezing behavior (not moving for more than 5 seconds at a time) after 2 and 7 days of exposure (Fig. 1a,b). However, at 14 days, fish exposed to elevated CO_2 spent significantly more time freezing ($p=0.043$) before and during exposure to a predator cue (Fig. 1e).

Having established that end-of-century levels of CO_2 in the water result in pronounced alterations of behavior, we tested if exposure to elevated CO_2 at the olfactory epithelium alone was sufficient to reduce the detection of odorants. We used electrophysiological recordings from peripheral sensory neurons of the olfactory system, allowing us to isolate peripheral olfactory responses from central brain processes. We measured changes in the activity of the olfactory nerve whilst exposing the olfactory epithelium to sea water containing ten different olfactory stimuli dissolved in either control or elevated CO_2 seawater, while fish were maintained under control CO_2 levels. We tested the olfactory nerve response of sea bass to a wide range of odorants: amino acids (L-cysteine, L-serine, L-alanine, L-arginine and L-glutamate), as odorants principally mediating food detection[21]; bile acids involved in chemically-mediated interactions between conspecifics and other teleost species (cyprinol sulphate) and potentially predatory shark species (scymnol sulphate)[22]; body fluids (intestinal fluid, bile from conspecifics, and alarm cue), potent chemical signals that can elicit behavioral responses vital for escape from/awareness of predators or recognition of conspecifics[23,24,25] (see Methods section for a full description). The amplitude of the response indicates the change in magnitude of the nerve activity in response to an odorant, and the detection threshold is defined as the concentration of odorant that produced a detectable response (above baseline). Overall, elevated CO_2 reduced the amplitude of the response for 6 out of the 10 odorants compared to control (Fig. 2), and increased the

detection threshold (i.e. reduced sensitivity) in 4 out of the 10 odorants (Fig. 3), but had no effect on the remainder (i.e. elevated CO_2 did not increase amplitude or sensitivity of the response to any of these odorants). Under elevated CO_2 the responses to L-alanine, L-arginine and L-glutamate, cyprinol sulphate, scymnol sulphate and alarm cue were up to 46% lower than those of controls (n=6-11 per odorant per treatment, p=0.027, p=0.040, p=0.028, p=0.011, p=0.012, p=0.018 respectively, Fig. 2). The thresholds of detection for L-cysteine (p=0.049), L-alanine (p=0.029) and L-glutamate (p=0.0047), and conspecific bile (p=0.029) were 2 to 5 fold higher in elevated CO_2 (Fig. 3). Therefore, under elevated CO_2 these odorants would need to be present in the water at concentrations up to 5 times greater than in current CO_2 conditions in order to be detected by sea bass.

The active space of a chemical is defined as the largest volume of water a fish can occupy that still contains a concentration of odorant at or above the olfactory threshold for detection[25,26]. This is a useful parameter to help estimate how much closer a fish would need to be (on average) to detect an odor-source under these elevated CO_2 conditions. We assumed a homogeneous distribution of the odorant in the water. Although this assumption may be simplistic for most natural environments (due to the constant movement of water associated with currents, tidal movements, etc.), it allows for a good estimate of the average change for most circumstances. The largest reduction in threshold of detection (5 fold) was for glutamate (Fig. 3). We calculated that the active space (i.e. a 3 dimensional volume) for glutamate detection would be reduced by 80%, which would translate into fish having to be 42% closer to the odorant source before detection occurred (i.e. based on the one dimensional radius of the three dimensional active space sphere; Fig. 3 - see Methods for calculation details and assumptions).

Lastly, RNA sequencing was used to elucidate the molecular mechanisms underpinning the negative effects of elevated CO_2 on sea bass olfaction. Sea bass were exposed for 2 and 7 days to either control (~450 µatm) or elevated CO_2 (~1000 µatm). Global gene transcription was measured in tissue samples taken from the olfactory epithelium (n=6) and the olfactory bulb (n=4) using an Illumina HiSeq 2500 platform. *De novo* reference transcriptomes were constructed for each tissue using the Trinity pipeline[27] (see Methods for full description). Transcript abundances were calculated using RSEM[28] and differences in gene transcription were determined using EdgeR[29] and a selection of scripts provided by Trinity.

Calcium/calmodulin-dependent protein kinase II beta 2 (CAMKII) directly regulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors, known to be involved in synaptic plasticity[30]. In the olfactory bulb, fish exposed to elevated CO_2 for 2 days showed a significant down-regulation of *camk2ga* (gene encoding CAMKII) and *nptxr* (encoding for a protein involved in synaptic plasticity and the clustering of AMPA receptors). This was followed by the downregulation of *gria1b* (homologous to a gene encoding AMPA glutamate receptors), downregulation of *map2k2a* (gene encoding mitogen activated protein kinase kinase involved in olfactory learning in the olfactory bulb in rats[31]), and the upregulation of *tmub1* (gene encoding a protein involved in the regulation of AMPA receptors at the cell surface) at 7 days. Additionally, genes involved in neurotransmitter re-uptake (*slc6a17* and *slc1a8*) including glutamate were downregulated in the olfactory epithelium in fish exposed to elevated CO_2 for 7 days. In the olfactory epithelium there was an upregulation of *slc4a8* (gene encoding the sodium/bicarbonate cotransporter) at 2 days, similar to that observed for long-term adjustments of bicarbonate (HCO_3^-) transport in the gills of marine fish in response to

elevated CO_2[32]. Four chemosensory G-protein coupled receptor gene families have been identified in teleosts: main olfactory receptors (ORs), trace amine-associated receptors (TAARs), and vomeronasal receptors 1 and 2 (VR1 and VR2)[33] and all of these were well represented in the olfactory epithelium transcriptome (see Supplementary Table S6). In the olfactory epithelium two OR genes (*or* and *or142)* were significantly down-regulated after 7 days of exposure; however, no OR genes were up-regulated. Interestingly, in the olfactory bulb two additional OR genes (*or* and *or120*), likely expressed in the axons of sensory neurons reaching the olfactory bulb, were also downregulated in fish exposed to elevated CO_2 for 7 days. In mammals, the presence of odorant receptor mRNA in the axons of sensory neurons is well established, and these mRNAs are likely involved in the wiring of the olfactory system[34]. These results suggest that fish exposed to elevated CO_2 did not activate compensatory molecular mechanisms to adjust for the loss of olfactory sensitivity measured using nerve recording, and that the wiring of the olfactory system might be affected by ocean acidification.

Genes involved in excitatory neurotransmission such as the nicotinic acetylcholine receptor (*chrna7)* and glutamate receptor (*gria1b*) were down-regulated in the olfactory bulb of fish exposed to elevated CO_2 levels for 7 days, while a gene involved in decreasing neuronal excitability (calcium-activated potassium channel, *kcnn3*) was up-regulated in the same tissue after 2 days of exposure. In the olfactory epithelium, fish exposed to elevated CO_2 for 7 days showed down-regulation of excitatory neurotransmission (*scn4ab, cacna2d*), and neuronal growth and development (*zak* and *efnb2a*). These results indicate mechanisms for decreased excitability of neurons in the olfactory epithelium and

bulb at both time points, and thus, a decrease in olfactory information being transmitted to higher brain centers.

Discussion

Elevated CO_2 affects behavior

Adult sea bass spawn offshore and the newly-hatched larvae must navigate back to the safety of coastal nursery habitats using their olfactory senses to home in on these sites and to avoid predators[35], and this life history strategy is shared by many fish species. This is the most vulnerable stage in the life cycle of sea bass being associated with high mortality from predation[35]. Here, we report that juvenile sea bass exposed to levels of CO_2 predicted for the end of the century demonstrate impaired behavior in response to a predator cue (Fig. 1). This response is similar to those reported for other fish species studied to date and, importantly, an increased effect was observed with exposure time[3,4,11,12]. However, in sea bass the baseline activity was lower after exposure to elevated CO_2 (Fig. 1), which is unlike most fish species studied to date (generally higher activity was reported after exposure to elevated CO_2)[36,37]. Although a recent study found no effects of elevated CO_2 on the routine swimming behavior of early juvenile sea bass[38], this could have been influenced by much higher control CO_2 conditions (585 μatm *vs* 430 μatm in our study). This higher level of CO_2 (585 μatm) is predicted to be reached during the mid 21st century, and behavioral impairments were reported in some species following exposure to this level of CO_2[39]. In our study, juvenile sea bass exposed to elevated CO_2 also spent more time freezing compared to those exposed to control CO_2 levels; this is consistent with previous findings showing that rockfish exposed to elevated CO_2 had elevated levels of anxiety compared to control fish[11].

Elevated CO_2 affects olfactory sensitivity

189 The decreased behavioral response to the predator odor was accompanied
190 by a decrease in olfactory sensitivity (either via response amplitude or detection
191 threshold) to 8 out 10 odorants tested, indicating that olfaction is generally impaired
192 in sea bass exposed to elevated CO_2. The sensitivity of some odorants was more
193 affected than that of others, suggesting that the quality of the perceived odor might
194 be altered in fish exposed to elevated CO_2. This may help to explain the
195 inappropriate (rather than simply inhibited) behavioural responses to complex
196 predator and home-reef odours previously described in the literature[3, 4]. Furthermore,
197 our results are consistent with studies in freshwater pink salmon (*Oncorhynchus*
198 *gorbuscha*) and marine shore crabs (*Carcinus maenas*) exposed to elevated CO_2
199 showing that olfactory sensitivity is impaired, even after prolonged exposure to
200 elevated CO_2, and can be restored within two hours or less of return to control
201 conditions[40,41], indicating that this effect persists during chronic exposure to elevated
202 CO_2, but is readily reversible. In freshwater fish and marine crabs exposed to
203 elevated CO_2/low pH the loss of response to some odorants, including alarm cue,
204 has been attributed to structural and functional changes of the chemical cues
205 themselves[19,41]. This is also a potential explanation for how elevated CO_2 affects
206 odorant-receptor binding in the current study.
207 We estimate that under elevated CO_2 sea bass must be up to 42% closer to
208 the odorant source than in current day conditions to allow detection (Fig. 3). This
209 would increase the risk of predation or decrease the ability to find food, resulting in a
210 direct impact on survival. This is consistent with the observation that coral reef fish
211 raised in elevated CO_2 show a 9-fold increase in mortality in the wild[4]. The activity of
212 sea bass exposed to elevated CO_2 was significantly reduced (Fig. 1) likely resulting
213 in reduced energetic costs. Importantly, this reduced activity would also further

reduce their chances of encountering odors in elevated CO_2 conditions. Therefore, if these changes in detection thresholds persist during longer term exposure to elevated CO_2 (as found in freshwater salmon[40]), these could have important ecological consequences at the population level, affecting communication with conspecifics, prey detection and, particularly, predator avoidance.

Elevated CO_2 affects gene expression

Only one recent study has investigated the effect of elevated CO_2 on global gene expression patterns in the brain of fish, and found that in juvenile spiny damselfish (*Acanthochromis polyacanthus*) genes associated with brain glucose, serine and glycine metabolism were differentially expressed in fish exposed to elevated CO_2[42]. Interestingly, in our study both electrophysiology and RNA-Seq results indicated that the response to glutamate was most affected by elevated CO_2. We show that in fish exposed to elevated CO_2 genes encoding CAMKII, MAPK kinase and AMPA glutamate receptors were downregulated, and genes associate with AMPA receptor cycling (*tmub1* and *nptx2*) were upregulated. These genes are involved in long term depression (a long-lasting decrease in synaptic strength), a process associated with a decline in learning and memory in higher brain centres[30]. The expression of genes involved in maintaining neuronal excitability (*chrna7, gria1b, scn4ab, cacna2d*) also decreased in fish exposed to elevated CO_2. A decrease in synaptic plasticity in the olfactory bulb and a decrease in neuronal excitability in the olfactory system suggest that less olfactory information was being sent to higher brain centers. Additionally, OR genes in the olfactory bulb were downregulated in fish exposed to elevated CO_2. These genes have been shown to be involved in the patterning of the olfactory bulb in mammals, particularly during the development of the olfactory system[34]. Interestingly, these findings are consistent

with impaired learning in responding to a predator odor in larval damselfish (*Pomacentrus amboinensis*) exposed to elevated CO_2[13], perhaps due to reduced olfactory information reaching higher brain centers, compromising learning and memory formation.

Novel physiological mechanism

We propose a novel mechanism based in the olfactory system to explain how elevated CO_2 alters the behavior of fish (Fig. 5). First, we show that elevated CO_2 can have a direct effect on the sensitivity of olfactory reception to various odorants in sea bass, likely by reduced affinity of odorant-receptor binding in the olfactory epithelium. Our electrophysiology data show that fewer impulses are sent to the olfactory bulb in response to most odorants, regardless of concentration. This would result in a decrease in the activity of olfactory bulb synapses, detected by a change in the timing and the frequency of calcium cycling in these neurons, a process that can lead to a decrease in synaptic plasticity[30]. Indeed, gene expression results show that sea bass exposed to elevated CO_2 downregulate genes involved in synaptic plasticity and maintaining the excitability of both peripheral olfactory receptor neurons and central olfactory bulb neurons, supporting our hypothesis. Therefore, we propose that under future levels of elevated CO_2 fish may sense less information through their olfactory receptors, and this would be compounded by less peripheral olfactory information being transmitted to higher brain centers. Additionally, we also found decreases in the expression of genes involved in in the wiring of the olfactory system, an important developmental process for juvenile fish. These physiological and molecular changes are consistent with the altered behavior observed in this study and others and have strong implications for fitness in the wild.

263 The mechanism of altered neurotransmitter function previously hypothesized to
264 explain the impairments of sensory-induced behaviors observed in coral reef fish is
265 limited to alterations at the level of the brain[43]. It proposes that extracellular acid-
266 base regulatory changes that fish undergo in response to exposure to elevated CO_2
267 lead to changes in gradients for HCO_3^- and Cl^- ions across neuronal cell membranes
268 in the brain. In turn these changes are suggested to interfere with the normal
269 functioning of the gamma-aminobutyric acid A ($GABA_A$) receptor, causing increased
270 excitation rather than inhibition of the nervous system and the observed downstream
271 behavioral impairments[18, 43]. However, not all fish are good acid-base regulators, and
272 some do not regulate extracellular pH at all when facing elevated CO_2
273 environments[44]. The mechanism proposed here is independent of any changes in
274 blood acid-base chemistry but is instead dependent on the external (seawater)
275 changes in CO_2/H^+. This raises the possibility that all fish species exposed to
276 elevated CO_2 are potentially susceptible to the direct impairment of peripheral
277 olfactory sensitivity proposed here, whereas the central brain impairment of sensory
278 behavior will principally be relevant to species that are good acid-base regulators.
279 An apparent discrepancy is that in some studies behavioral abnormalities
280 previously shown for fish exposed to ocean acidification are not evident for the first
281 24 hours of exposure[4]. However, these previous observations are based on fish
282 exposed to strong odors, probably well above the threshold of detection[3,4,43]. Thus,
283 even a 50% reduction in the olfactory sensitivity at these high odorant concentrations
284 would not prevent fish from smelling these strong predator odors, giving rise to some
285 form of behavioral response under elevated CO_2 conditions. By contrast, the
286 peripheral mechanism proposed here would impair olfaction following any duration of
287 elevated CO_2 conditions, particularly when odorants are close to their detection

threshold, a more realistic scenario in a natural environment. Secondly, the behavioral responses documented previously are downstream of the central brain GABA-regulated processes that should only be impaired secondary to acid-base regulation and changes in blood chemistry[45]. It is also important to recognize that the two models (the one proposed here based in the olfactory system and the previously proposed impairment of GABA receptor function) are not mutually exclusive. Indeed, it seems likely that they would operate together during exposures lasting longer than 24 h, in particular for acid-base regulators, impacting sensory behavior through two distinct physiological mechanisms and ultimately impairing fitness.

Conclusions

Recent studies, including ours, indicate that behavioral responses persist, or become more pronounced, with prolonged experimental exposure to elevated CO_2[4,12] and in fish that live in naturally high CO_2 environments (near CO_2 seeps)[46]. Additionally, it is not known if the relatively fast change in CO_2 predicted for this century would allow sea bass and other fishes to acclimate or adapt to a high CO_2 world, but one generation is apparently not enough to mitigate the effects of elevated CO_2[47]. We propose that the impairment of sensory behavior is induced via not one, but two complementary physiological mechanisms, acting on the olfactory system and on the GABA receptor function in the brain. In essence, fish are impacted at two distinct levels of the sensory-behavioral system, both at the periphery and the central nervous system affecting their behavior. This suggests that complete adaptation may require phenotypic modification at both of these targets of CO_2 exposure. In turn this could either increase selection pressure on this sensory pathway or increase the time required for selection compared to if there was only one target mechanism, and thus complicate predictions about the length of time required for adaptation to occur.

313 Ultimately, it is becoming clear that an elevated CO_2 environment has the potential
314 for major negative impacts on olfactory-mediated behavior of fish across a wide
315 range of habitats and latitudes. This highlights the potential for ecologically
316 significant population-level impacts on fishes, and perhaps other marine fauna,
317 including on economically and ecologically important species.

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Author contributions

CSP and RWW designed the behavior experiments. CSP performed the experiments and analyzed those data; CSP, PCH and RWW designed the electrophysiology study, CSP and PCH performed the electrophysiology experiments. CSP, TMUW, RvA, and EMS designed the transcriptomics experiments, CSP performed the experiments and constructed the libraries. CSP performed the bioinformatics analysis and interpreted the results with help from TMUW, RvA, and EMS; all authors contributed to and provided feedback on various drafts of the paper.

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Methods

1.1 Fish maintenance

Juvenile European sea bass (*D. labrax*; 4–8 g, 7 cm total length) were obtained from Ecloserie Marines de Gravelines, France and initially held within a stock tank containing recirculating artificial seawater (15°C) in the University of Exeter Aquatic Resource Centre. Photoperiod was controlled (12:12 light:dark) and fish were fed twice daily *ad libitum* with Perla MP pellets (Skretting, Shay Lane, Longridge, Preston, UK).

1.2 Exposure to simulated ocean acidification

All experiments were conducted under approved protocols according to the UK Home Office regulations for use of animals in scientific procedures, and approved by the University of Exeter Ethics committee. Sea bass were exposed to simulated ocean acidification for 2, 7 and 14 days as described previously[48]. For water chemistry parameters during exposure for behavioral and the transcriptomics experiments see Supplementary Table S1.

1.3 Behavioral testing

Fish were fed 1% body mass of Perla MP pellets (Skretting, Shay Lane, Longridge, Preston, UK) before behavioral experiments to prevent hunger from over-riding behavioral responses to a predator[49]. Sea bass were then transferred by scoop (without air exposure) to a flume (60 cm x 16 cm x 8 cm) using flow-through control or elevated CO_2 (same as exposure; see Supplementary Table S2). Sea bass were left for 30 min in the flume as preliminary experiments indicated this was sufficient time for them to resume normal activity and behavior after transfer. Fish behavior was recorded from above using a video camera (Sony Handycam, DCR-SR190). The bottom of the flume had a black grid in order to quantify activity. Activity was

recorded for 5 min to determine baseline activity, after which diluted (1:1,000,000) monkfish (*Lophius piscatorius*) bile was added to the inflowing water for a further 5 min. Two fish were tested at the same time (one from each treatment) and they were randomly assigned.

Video analysis was used to quantify the number of squares visited by each fish in the minute before monkfish bile and during the 5 min in which monkfish bile was present. Video analysis was carried out blind regarding CO_2 treatment. The total number of seconds spent freezing was quantified (expressed as % of time) in the 5 min prior to and during presence of monkfish bile. A fish was considered to be freezing when it spend more than 5 sec without moving.

Statistical analysis

To test for the effects of treatment and time (day) on baseline activity and change in activity a two-way analysis of variance was performed (SigmaPlot, version 9.2). A Holm-Sidak *post hoc* test was used to test for differences from control and between different times. To test for the effects of treatment and presence of predator odor on freezing behavior a two-way repeated measures analysis of variance was performed, followed by Holm-Sidak *post hoc* test when significant differences were detected (at 7 and 14 days). A significance level of $P < 0.05$ was used throughout the analysis.

1.4 Transcriptomics

RNA extraction, library preparation and sequencing

Fish were humanely sacrificed on day 2 and 7 of the exposure period (see above) by a lethal dose of benzocaine (0.5 g L-1; Sigma-Aldrich). Brains and olfactory epitheliums were dissected and snap frozen in liquid nitrogen and stored at -80°C

prior to transcript profiling. Transcript profiling was conducted in the brain and olfactory epithelium of 6 and 4 fish per treatment group, respectively.

RNA was extracted using an RNeasy mini kit (QIAGEN, Catalog # 74104), and on-column DNAse treatment was performed, according to the manufacturer's instructions. The concentration, purity and integrity of RNA were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). All RNA input to library construction was of high quality with A_{260}/A_{280} and A_{260}/A_{230} ratios > 2.0 and RIN scores > 8.5. ERCC spike-in control mixes (Ambion) were added to all individual RNA samples, according to the manufacturer's instructions to allow for analysis of the accuracy of the transcript quantification and dynamic range (Supplementary Table S3-S4). cDNA libraries for all samples were then prepared using the NextFlex Rapid RNA-Seq Kit (Cat # 5138-02), multiplexed with 12 and 16 samples per lane for the olfactory epithelium (OE) and bulb (OB), respectively (4 lanes total). The samples were sequenced using an Illumina HiSeq 2500 in standard mode, to generate 100 bp paired reads. Sequencing of the OE and OB produced 1007 and 349 million paired 100 bp reads, respectively, averaging 41.9 and 21.8 million reads per library, respectively.

Data analysis

All analyses were carried out on a local server running the NEBC Bio-Linux 7 environment[50]. Raw sequence reads were quality-trimmed using Trimmomatic[51] (v0.32). The first 12 bp were removed from each read (to remove 5' bias caused by non-random hexamer priming) and a 4-base wide sliding window was used to cut when the average Phred score quality per base was below 20. Furthermore, bases were trimmed of the end of the reads when the Phred score quality was less than 15

564 and reads shorter than 36 bp were discarded. Digital normalization was performed to
565 remove highly duplicated reads using the normalize-by-median.py script part of the
566 khmer package described by Brown et al.[52], with the recommended k-mer value of
567 20 and a coverage threshold of 30. Following digital normalization, a total of 69.4
568 and 47.2 million paired reads originating from all the OE and OB sequence libraries,
569 respectively, were retained and input into the *de novo* transcriptome assemblies. The
570 assemblies were constructed using Trinity[53] (version 2.1.1) and the default
571 parameters, specifying a minimum contig length of 200 bp (see Supplementary
572 Table S5 for comparative summary statistics for each assembly). All transcripts in
573 the final assemblies were annotated using Blastx[54] against the Ensembl peptide
574 databases[55] (Release 79; March 2015) using an E-value cut-off of $1e^{-15}$ and 'best
575 hits' were assigned to transcripts in the following preferential order to facilitate
576 assigning Gene Ontology (GO) terms for subsequent functional annotation analysis:
577 zebrafish (*Danio rerio*); human (*Homo sapiens*) and mouse (*Mus musculus*);
578 stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), Japanese pufferfish
579 (*Takifugu rubripes*), tilapia (*Oreochromis niloticus*), green spotted puffer fish
580 (*Tetraodon nigroviridis*), cod (*Gadus morhua*) and spotted gar (*Lepisosteus*
581 *oculatus*). Additional annotation was performed using Diamond blastx[56] (v0.8.22.84;
582 E-value $< 1e^{-10}$) against the full NCBI nr protein database (March 2016). Transcripts
583 that were not annotated or that showed similarity to non-metazoan genes only
584 (n=826 and n=964 for the olfactory epithelium and olfactory bulb, respectively) were
585 removed from the assembly prior to differential expression analysis, using MEGAN
586 5[57] (version 5.11.3, January 2016). All four families of olfactory receptors were well
587 represented in the olfactory epithelium transcriptome (Supplementary Table S6).

588 Reads from individual samples were mapped against the Trinity transcriptome
589 assemblies for each tissue and transcript abundances were calculated using RSEM
590 and the align_and_estimate_abundance.pl script supplied by Trinity (version 2.1.1).
591 Differential expression analysis was carried out at the gene level using EdgeR and
592 the run_DE_analysis.pl script provided by Trinity. Genes were considered
593 differentially expressed with a FDR < 0.1 (Benjamini-Hochberg correction; see
594 Supplementary File 2 and Figures S2-S4). Hierarchical clustering was performed on
595 all differentially expressed genes using the analyze_diff_expr.pl script. Functional
596 analysis was then performed for differentially expressed genes from each tissue
597 using the Database for Annotation, Visualization and Integrated Discovery[58] (DAVID
598 v6.8, Oct 2016), using the zebrafish gene identifiers and the *de novo* transcriptomes
599 for each tissue as a background (see Supplementary Tables S7-S10). Kyoto
600 Encyclopedia of Genes and Genomes (Kegg) pathways (http://genome.jp/kegg/) and
601 Gene Ontology (GO) terms (http://geneontology.org/) for Biological Process, Cellular
602 Component and Molecular Function were considered significantly over-represented
603 when adjusted P < 0.1 (Benjamini-Hochberg).

604 *qRT-PCR validation*

605 RNA-Seq expression was validated using Quantitative Realtime PCR (qRT-PCR) for
606 a selection of key genes that were shown to be differentially expressed in the
607 olfactory epithelium using RNAseq (see Table S11). We used a combination of the
608 same samples used for the RNA-Seq and additional replicates from the same
609 experiments (n=5-8 replicates per treatment). Primers were designed for the
610 assembled transcripts using OligoArchitect Online (Sigma-Aldrich) and blasted using
611 the NCBI BLAST tool to verify specificity. RNA concentration and purity were
612 assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies,

Wilmington, USA). cDNA was synthesized according to manufacturer's instructions from 2 µg of total RNA treated with RQ1 DNase (Promega, Southampton, UK) using random hexamers (MWG-Biotech) and M-MLV reverse transcriptase (Promega). cDNA was diluted 1:2 and RT-qPCR was performed in triplicate using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green chemistry (Bio-Rad). Each reaction was performed in a total volume of 15 µl. PCR conditions consisted of 10 minutes at 95 °C, followed by 40 cycles of 10s at 95°C and 30s at the optimized annealing temperature for each primer pair. High resolution melt analysis (60-95°C, in 0.5 °C increments) was then performed to verify the presence of single amplification products of the expected size and Tm. A template-minus negative control was run in triplicate on each plate to verify the absence of cDNA contamination, and replicates displaying non-specific amplification were removed from the analysis. Annealing temperature for each primer pair was optimized by performing a standard curve. The linear correlation (R^2) between the mean Cq and the logarithm of the cDNA dilution was > 0.98 in each case, and efficiencies were between 1.92-2.11. The primer sequences, PCR product sizes, annealing temperatures and PCR efficiencies for each primer pair are shown in Table S11. Efficiency-corrected relative expression levels of each target gene were determined according to Pfafl[59] and by normalizing to the control gene, *rpl8*. This control gene was chosen because it remained constant across all treatments and tissues in the RNA-Seq experiments and has been widely used as a control gene for qPCR experiments in fish[60]. Unpaired two-sample t-tests were used to test for significant differences between treatments.

1.5 Electrophysiology

Experimental animals

638 Fish care and experimentation complied with the guidelines of the European
639 Union Council (86/609/EU) and Portuguese legislation for the use of laboratory
640 animals under a ‘‘Group-1’’ license issued by the Veterinary General Directorate of
641 the Ministry of Agriculture, Rural Development and Fisheries of Portugal. Juvenile
642 European sea bass (*Dicentrarchus labrax*; 16.6 - 36.0 g) were obtained as 2 month-
643 old larvae from the Ifremer experimental aquaculture station in Palavas-las-Flots,
644 France, and raised in flow-through natural sea water from the Ria Formosa (sand-
645 filtered and UV sterilized, ~36 psu) at the Ramalhete Aquatic Facilities, Faro,
646 Portugal. Sea bass were transferred to Laboratório Experimental de Organismos
647 Aquáticos and held in recirculated natural sea water (as above) at 20°C on a 12 h
648 light : 12 h dark photoperiod for at least two weeks before the start of experiments.

649 *Choice of olfactory odorants*

650 We tested the effect of acutely elevated CO_2 on the olfactory sensitivity of sea
651 bass to amino acids, as odorants principally mediating food detection[21]. Bile acids
652 are cholesterol derivatives secreted in the bile fluid of vertebrates that aid in the
653 digestion and absorption of lipids and lipid-soluble vitamins[61, 62]. In fish they are also
654 potent olfactory stimuli, which may be involved in chemically-mediated interactions
655 both within and between species[63]. Cyprinol sulphate is a widely produced bile acid
656 in cyprinids but also found in other teleosts[61] and scymnol sulphate is a major
657 component of elasmobranch bile[61]. The body fluids of fish are complex mixtures that
658 can contain potent chemical signals[1,64,65], including alarm cues released from
659 damaged skin of some teleost fish that elicit behavioral responses vital for nearby
660 conspecifics to escape from, or be aware of, predators[23,24]. Therefore, we measured
661 the effect of elevated CO_2/H^+ on the olfactory responses of sea bass to conspecific
662 bile, intestinal fluid and damage-released alarm cue.

663 *Collection of body fluids and alarm cue*

664 Body fluids (bile and intestinal fluid) were collected from three male and three
665 female sea bass (146-205 g) that were being sampled for other experiments. Once
666 collected, the samples were kept on ice until return to the lab, then centrifuged, all
667 were pooled and frozen (-20°C) into aliquots until use. For alarm cue four sea bass
668 were killed by a sharp blow to the head. Care was used not to touch the flanks of the
669 fish. Using a sharp scalpel, twelve 1 cm superficial cuts were made to one flank of
670 each fish. The flank was rinsed with charcoal-filtered sea water. This was then either
671 immediately frozen in aliquots for later use (no longer than 48 h later) or diluted for
672 immediate use.

673 *Olfactory nerve recording*

674 Sea bass were anesthetized in aerated natural seawater containing 300 mg l^{-1}
675 MS222 (ethyl-3-aminobenzoate methanesulphonate salt; Sigma-Aldrich, Spain)
676 buffered with 0.6 g.l^{-1} sodium bicarbonate, until response to tail pinch had stopped.
677 An intramuscular injection of gallamine triethiodide (Sigma-Aldrich, Spain; 3 mg kg^{-1}
678 in 0.9% NaCl) was then given[66,67,68]. The sea bass were then placed in a padded V-
679 support and the gills flushed with aerated natural sea water containing 150 mg l^{-1}
680 MS222 (also buffered as above). The electrodes were placed in the olfactory nerve
681 (near the bulb) in a place that gave maximal response to 10^{-4} M L-cysteine.
682 Olfactory nerve activity was recorded using tungsten micro-electrodes (0.1 MΩ,
683 World Precision Instruments, UK) as previously described[26] (Supplementary Fig S5).
684 The fish was connected to earth via a copper wire inserted in the flank. The raw
685 signal was amplified (20,000x; AC pre-amplifier, Neurolog NL104; Digitimer Ltd.,
686 Welwyn Garden City, UK), filtered (high pass: 200 Hz, low pass: 3,000 Hz; Neurolog
687 NL125, Digitimer Ltd.) and integrated (time constant 1 s; Neurolog NL703, Digitimer

688 Ltd.). Raw and integrated signals were digitized (Digidata 1440A, Molecular Devices,
689 Sunnyvale, CA, USA) and recorded on a PC running AxoScope™ software (version
690 10.2, Molecular Devices).

691 At all times, the gills of the fish were flushed with sea water equilibrated with
692 atmospheric air (i.e. control conditions, pH 8.15) containing anesthetic (see above);
693 therefore, only the olfactory epithelium (also known as the rosette) experienced
694 elevated CO_2 conditions. Charcoal-filtered natural sea water was either bubbled with
695 air (control, pH 8.15 ± 0.01, 476 ± 14 µatm) or CO_2 (pH 7.82 ± 0.01, 1122 ± 19 µatm)
696 until the desired pH_{NBS} was reached (see Water Chemistry section for details;
697 Supplementary Table S12). The charcoal-filtered sea water (control or elevated CO_2)
698 was used to make up the odorant solutions and to superfuse the olfactory epithelium
699 during experiments. Amino acid and bile acid solutions were prepared from frozen
700 aliquots of 10^{-2} M, while body fluids were prepared from frozen aliquots and all were
701 diluted in charcoal-filtered sea water (either control or elevated CO_2) immediately
702 prior to being used. The order of testing of the odorants was randomized, but each
703 odorant was always given from lowest to highest concentration (10^{-8} M to 10^{-3} M).

704 All responses to a given stimulus (except alarm cue) were paired: responses
705 were first recorded while the olfactory epithelium was superfused with control sea
706 water, followed by responses to the odorant made up in elevated CO_2 while the
707 olfactory epithelium was also superfused with elevated CO_2 sea water. Initial
708 measurements were performed in control conditions, followed by elevated CO_2, and
709 then control again and the response was reversible (see Supplementary Fig S6).
710 Therefore, multiple stimuli were tested on the same fish under both control and
711 elevated CO_2 conditions (see Supplementary Fig S7). For testing the responses to
712 alarm cue, half of the responses were measured first under control and then under

713 elevated CO_2 sea water and the other half were measured in the reverse order to
714 account for the rapid degradation of the alarm cue (see Supplementary Fig S8).
715 Each odorant was added through a gravity fed three-way-valve (at a rate of 6 ml min^{-1}
716). A blank stimulus, treated in the same way as the odorants, was recorded at the
717 beginning and end of each group of samples. All integrated response amplitudes
718 were normalized to the amplitude of the integrated response to 10^{-4} M L-cysteine
719 (the 'standard') made up in control charcoal-filtered sea water. Responses to blanks
720 and standards were recorded regularly at the beginning and end of each group of
721 samples (every 3 - 5 samples) throughout the recording session. Each stimulus was
722 applied for 4 seconds, with at least 1 minute between odorants to allow complete
723 recovery of the receptors[26].

724 *Water chemistry measurements*

725 Salinity and temperature were measured using a Thermo Scientific 'Orion Star
726 A329' (Thermo Fischer Scientific, Inc., Waltham, MA, USA) probe. Charcoal-filtered
727 sea water was previously equilibrated with atmospheric air, and then for the
728 experiments this seawater supply was either bubbled with air (control) or elevated
729 CO_2 until the desired pH_{NBS} was reached (pH meter Model HI 8314, Hanna
730 Instruments, Leighton Buzzard, UK; pH probe Model pHC2401, Radiometer
731 Analytical, Lyon, France). Sea water samples (12 ml) from both treatments were
732 collected and were preserved using standard methods[69], which were then stored at
733 4°C for dissolved inorganic carbon (DIC) analysis as previously described.

734 *Calculations*

735 The 'active space' of an odorant was calculated by dividing the release rate of
736 that odorant by the predicted detection threshold for it[25]

737 $Active\ space = \frac{Release\ rate}{detection\ threshold}$ or $V = \frac{X}{Y}$ (1)

For a 5 fold increase in detection threshold (e.g. glutamate)

$V(CO_2) = \frac{X}{5Y}$ or $V(CO_2) = \frac{V}{5}$ (2)

where, V(CO_2) is the active space in elevated CO_2 assuming no change in release rate.

We assumed a homogeneous distribution of the odorant in the water. Although this assumption might be representative of natural environments (due to tidal movements, wave action etc.), it represents a pragmatic and best estimate of the average impact. Based on this assumption we calculated the maximum distance from source for odorant detection:

$V = \frac{4}{3}\pi r^3$ (3),

where V is the active space and r is the distance from the odorant source.

$r = \sqrt[3]{\frac{3V}{4\pi}}$ in control or $r = \sqrt[3]{\frac{3V}{4\pi}}$ or $r = 0.62 * \sqrt[3]{V}$ and

$r = \sqrt[3]{\frac{3V/5}{4\pi}}$ in high CO_2 or $r = \sqrt[3]{\frac{3V}{20\pi}}$ or $r = 0.36 * \sqrt[3]{V}$ (4)

Therefore, r will decrease to 58% in elevated CO_2 and fish would have to be 42% closer.

Data and statistical analysis

All statistical analyses and calculations were carried out on normalized data. Detection thresholds were determined from the intercept with the x-axis of linear regression fit to the linear part of individual dose response curves for each odorant and each treatment [64, 70] (SigmaPlot, version 9.2). To test for the effects of concentration and treatment a two-way repeated measured analysis of variance was performed for all the odorants (SigmaPlot, version 9.2). A Holm-Sidak *post hoc* test was used to test for differences from control and between different concentrations.

761 Paired Student's *t*-tests were used to test for differences between treatments in
762 detection thresholds using Microsoft Excel. A significance level of $P < 0.05$ was used
763 throughout the analysis.
764
765 *Data Availability*
766 Sequence datasets are available through the NCBI database
767 (https://www.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi?subid2311090) and include
768 the individual gene expression sample files. Water chemistry, behaviour and
769 electrophysiology data are available through Pangaea
770 (https://doi.pangaea.de/10.1594/PANGAEA.884674).
771 *Gene expression results*
772 After 2 days of exposure to elevated CO_2 in both the olfactory bulb and the olfactory
773 epithelium there were 23 and 24 differentially expressed genes, respectively
774 (Supplementary Fig S2). After 7 days of exposure to elevated CO_2 there were 73
775 and 71 differentially expressed genes in the olfactory epithelium and bulb,
776 respectively (Supplementary Fig S2). Expression patterns of all five genes used for
777 validation using qRT-PCR were in agreement with those obtained with RNAseq
778 (Table S12), with three of these being significant ($p<0.05$) and two being marginally
779 not significant ($p = 0.05 - 0.12$, *tmp4* and *klc8*).

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Figure Captions

Figure 1. Behavior responses of European seabass (*Dicentrarchus labrax*) to a 5 min exposure to a predator odor (monkfish bile). Behaviour recorded after exposure to control (~420 µatm) or elevated CO_2 (~950 µatm) for 2, 7, and 14 days. **a**, baseline activity after 2, 7, and 14 days exposure to control and elevated CO_2. **b**, change in activity before and during the first minute of exposure to predator odor (dilution 1:1,000,000). Period of time spent freezing before and during 5 min exposure to predator odor after 2 (**c**), 7 (**d**), and 14 (**e**) days of exposure. Values are means ± s.e.m. Asterisks indicate statistically significant differences compared to control data obtained before exposure to predator odor. Crosses indicate statistical significance between control and elevated CO_2 treatments ($p < 0.05$).

Figure 2. Elevated CO_2 decreases the olfactory sensitivity of European sea bass to amino acids, bile acids and body fluids. **a**, L-serine (N=6). **b**, L-cysteine (N=6). **c**, L-alanine (N=8). **d**, L-arginine (N=6). **e**, L-glutamate (N=6). **f**, cyprinol sulphate (N=10). **g**, scymnol sulphate (N=10). **h,** intestinal fluid dilutions (N=6). **i,** conspecific bile dilutions (N=6). **j**, alarm cue dilutions (N=8). Responses measured under control (blue) (pH 8.15 ± 0.01, 476±14 µatm) and elevated CO_2 (orange) (pH 7.82 ± 0.01, 1122±19 µatm). Values are expressed as % of the response to 10^{-4} M L-cysteine and represented as mean ± s.e.m. Different letters indicate significant differences between the response to different concentrations of odorants ($p < 0.01$). † denotes differences between treatments ($p < 0.05$). See online Supplementary Fig S7 for raw traces of these responses.

Figure 3. Acute exposure of European seabass to elevated CO_2 (~1000 µatm) decreases the amplitude of the olfactory response and increases the detection threshold of several odorants tested. Elevated CO_2 reduces the active space (represented by the blue sphere) of an odor by up to 80% (represented by the yellow dashed line) and the distance to a detectable odor source (arrow) by up to 42% in European sea bass. This suggests potentially drastic consequences on their ecology and survival (see Methods for calculations and assumptions, and Fig. 2 for amplitude response curves). Asterisks and crosses indicate statistically significant differences from the control group ($p < 0.05$) in detection threshold and amplitude, respectively. NS, not significant. Sea bass image Kovalevska/shutterstock.com

Figure 4. Differential regulation of genes in the olfactory epithelium and olfactory lobe of European sea bass exposed to control and high CO_2. Genes involved in neuronal growth (*efnb2a*) and development (*zak*) were significantly down-regulated in the olfactory epithelium. Additionally genes encoding for ion channels (*scn4*, *cacna2, chrna7 and kcnn3*) responsible for maintaining cell excitability were also down-regulated in both the olfactory epithelium and the bulb. In the olfactory bulb there was also down regulation of glutamate ionotropic receptors (AMPA), mitogen activated protein kinase kinase (map2k2) and CAMKII indicative of long term depression (process involved in decreased synaptic plasticity). Moreover, olfactory receptor genes were downregulated in both the olfactory epithelium and the bulb, indicating no compensatory mechanism for loss of olfactory function and changes in the wiring of the olfactory system in juvenile sea bass. Arrows represent direct pathways of activation, and T bars represent direct pathways of repression.

Note that the axons of the olfactory sensory neurons in the epithelium synapse with neurons in the olfactory bulb.

Figure 5. Proposed mechanism of action of CO_2-induced ocean acidification on fish behavior via the olfactory pathway. Ocean acidification has an acute effect on the binding of odorants to their receptors, decreasing both detection threshold and amplitude of the response. Long term exposure to high CO_2 decreases cell and neuron excitability, indicating less olfactory information is being transmitted from the olfactory epithelium to higher brain centers. In combination with a decrease in synaptic plasticity, this altered gene expression can affect behavior and learning in fish.

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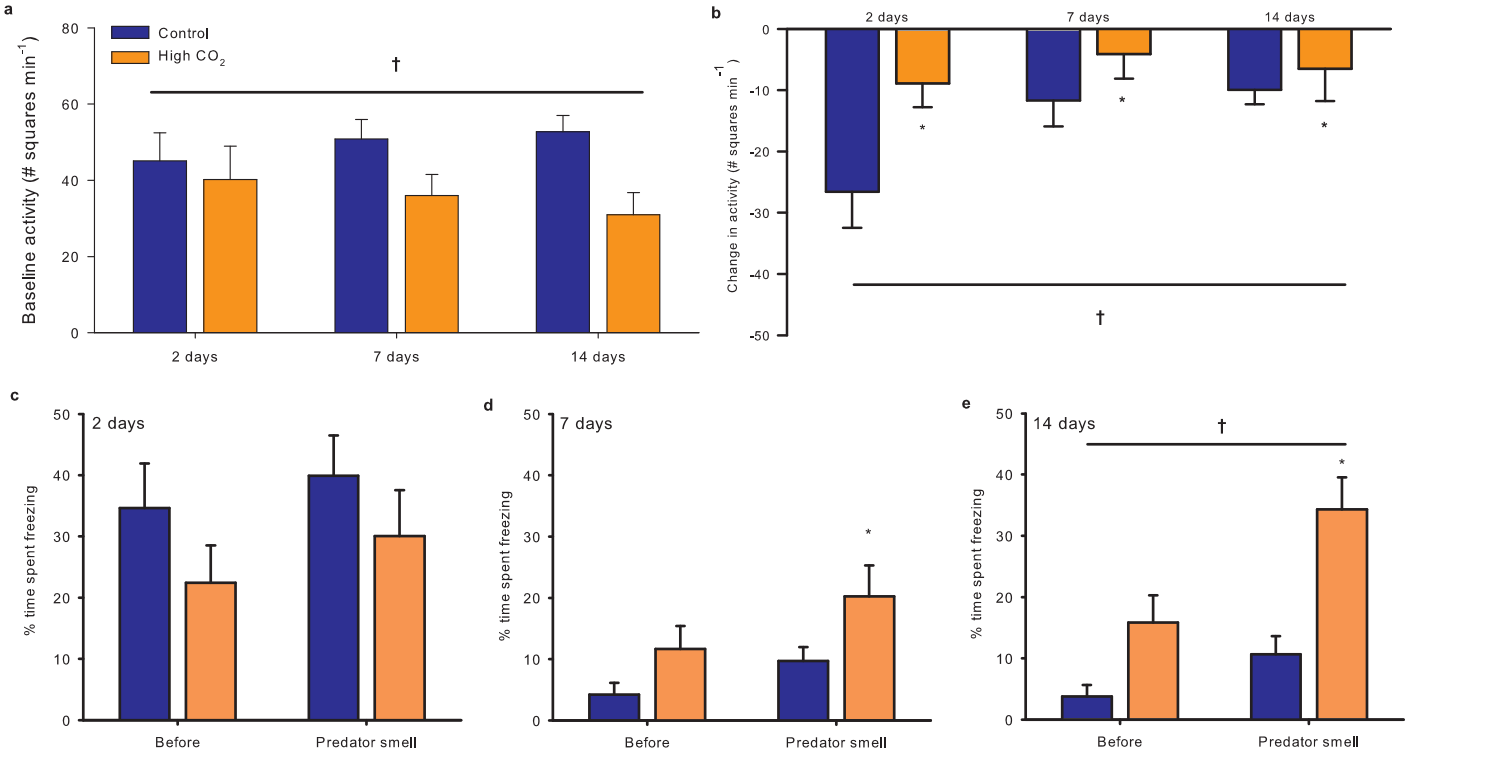
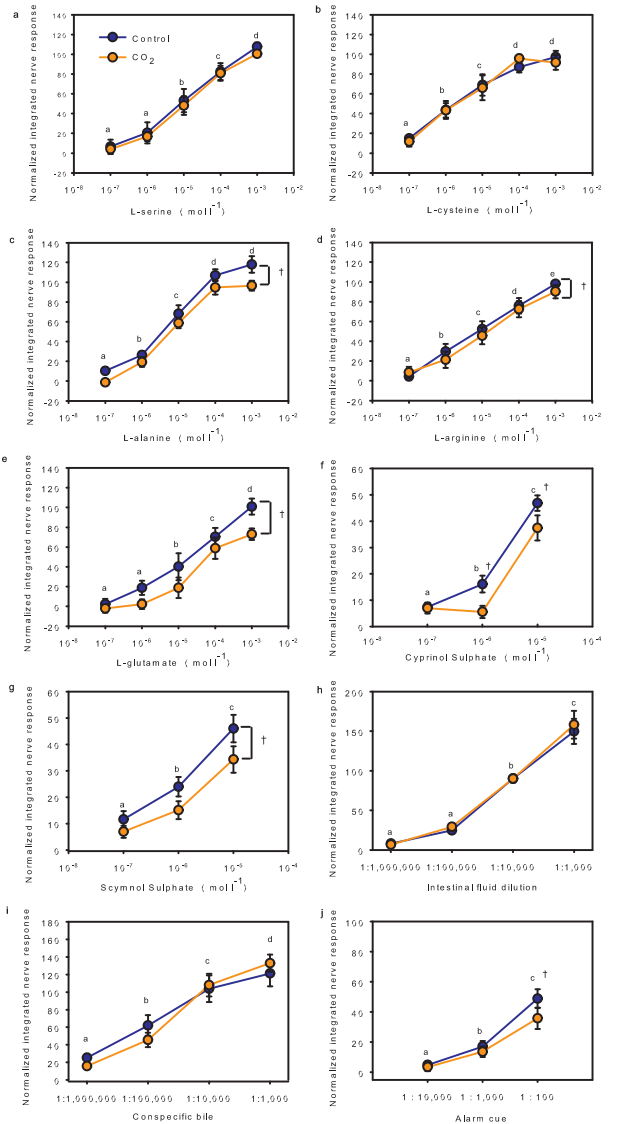
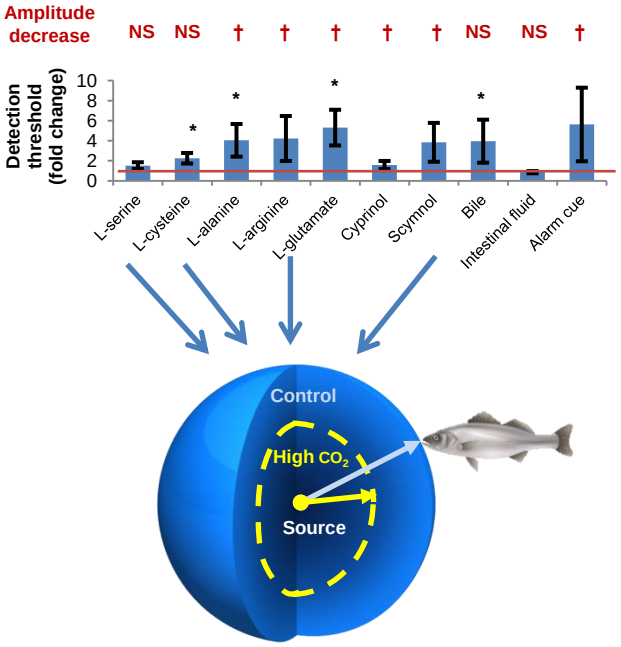
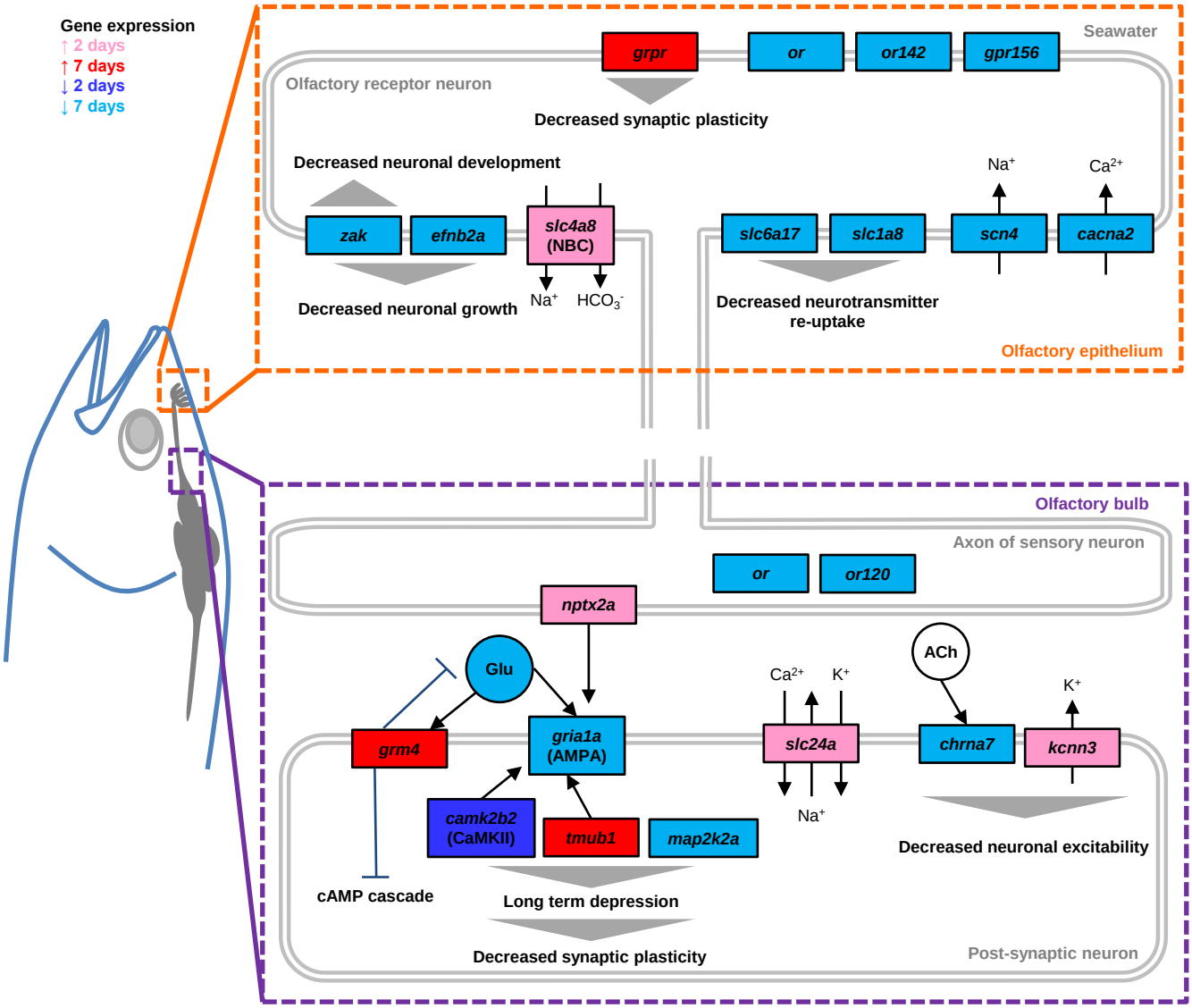
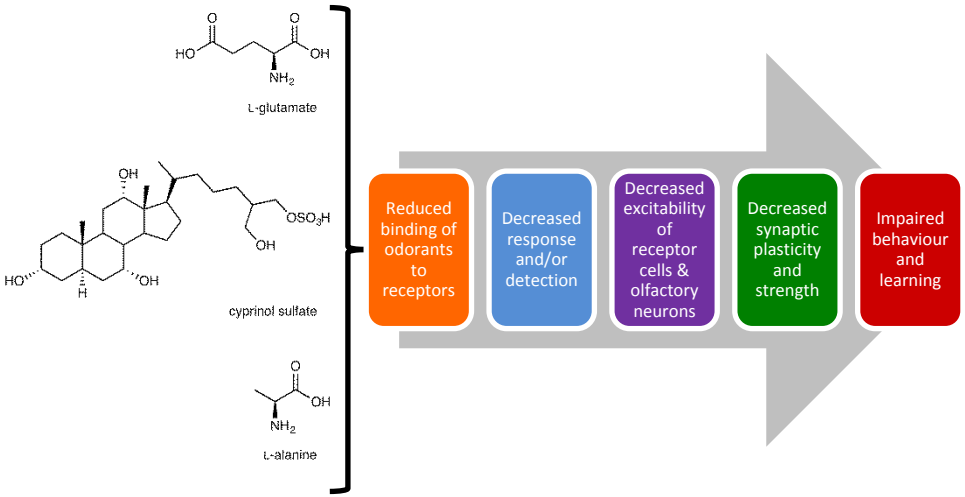

a
Control
High CO2
Baseline activity (# squares min-1)
80
60
40
20
0
†
2 days
7 days
14 days
b
2 days
7 days
14 days
Change in activity (# squares min-1)
0
-10
-20
-30
-40
-50
*
*
*
†
c
2 days
% time spent freezing
50
40
30
20
10
0
Before
Predator smell
d
7 days
% time spent freezing
50
40
30
20
10
0
*
Before
Predator smell
e
14 days
†
*
% time spent freezing
50
40
30
20
10
0
Before
Predator smell


a
Normalized integrated nerve response
Control
CO2
L-serine (mol l-1)
b
L-cysteine (mol l-1)
c
L-alanine (mol l-1)
d
L-arginine (mol l-1)
e
L-glutamate (mol l-1)
f
Cyprinol Sulphate (mol l-1)
g
Scymnol Sulphate (mol l-1)
h
Intestinal fluid dilution
i
Conspecific bile
j
Alarm cue


Amplitude decrease
NS
NS
†
†
†
†
†
NS
NS
†
Detection threshold (fold change)
10
8
6
4
2
0
*
*
*
*
L-serine
L-cysteine
L-alanine
L-arginine
L-glutamate
Cyprinol
Scymnol
Bile
Intestinal fluid
Alarm cue
Control
High CO_2
Source


Gene expression
↑ 2 days
↑ 7 days
↓ 2 days
↓ 7 days
Seawater
Olfactory receptor neuron
grpr
or
or142
gpr156
Decreased synaptic plasticity
Decreased neuronal development
zak
efnb2a
slc4a8 (NBC)
Na+
HCO3-
Decreased neuronal growth
Na+
Ca2+
slc6a17
slc1a8
scn4
cacna2
Decreased neurotransmitter re-uptake
Olfactory epithelium
Olfactory bulb
Axon of sensory neuron
or
or120
nptx2a
Glu
grm4
gria1a (AMPA)
camk2b2 (CaMKII)
tmub1
map2k2a
cAMP cascade
Long term depression
Decreased synaptic plasticity
Ca2+
K+
slc24a
Na+
ACh
chrna7
K+
kcnn3
Decreased neuronal excitability
Post-synaptic neuron


L-glutamate
cyprinol sulfate
L-alanine
Reduced binding of odorants to receptors
Decreased response and/or detection
Decreased excitability of receptor cells & olfactory neurons
Decreased synaptic plasticity and strength
Impaired behaviour and learning