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

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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 based in the olfactory system which helps explain altered behavior under elevated CO_2. We combine electrophysiology and high throughput sequencing with behavioral experiments to investigate how elevated CO_2 affects the olfactory system of European sea bass (*Dicentrarchus labrax*), an economically important species. Under elevated CO_2 (~1000 μatm) fish need to 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. These findings correlated with a 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 results contrast with, but complement, the previously proposed mechanism of impaired neurotransmitter (γ-aminobutyric acid) function, and demonstrate that both the olfactory system and central brain function are compromised by elevated CO_2 in the oceans, with potentially major negative impacts on fish globally.

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 most 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 Supplementary materials for a full description of the methods). 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 Supplementary Information 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 Supplementary Information for method 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 (Supplementary Information). 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 *efnb2b*). 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 the behavior of sea bass

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 has an acute effect on the olfactory sensitivity of sea bass

The decreased behavioral response to the predator odor was accompanied by a decrease in olfactory sensitivity (either via response amplitude or detection threshold) to 8 out 10 odorants tested, indicating that olfaction is generally impaired in sea bass exposed to elevated CO_2. The sensitivity of some odorants was more affected than that of others, suggesting that the quality of the perceived odor might be altered in fish exposed to elevated CO_2. This may help to explain the inappropriate (rather than simply inhibited) behavioural responses to complex predator and home-reef odours previously described in the literature[3, 4]. Furthermore, our results are consistent with studies in freshwater pink salmon (*Oncorhynchus gorbuscha*) and marine shore crabs (*Carcinus maenas*) exposed to elevated CO_2 showing that olfactory sensitivity is impaired, even after prolonged exposure to elevated CO_2, and can be restored within two hours or less of return to control conditions[40, 41], indicating that this effect persists during chronic exposure to elevated CO_2, but is readily reversible. In freshwater fish and marine crabs exposed to elevated CO_2/low pH the loss of response to some odorants, including alarm cue, has been attributed to structural and functional changes of the chemical cues themselves[19, 41]. This is also a potential explanation for how elevated CO_2 affects odorant-receptor binding in the current study.

We estimate that under elevated CO_2 sea bass must be up to 42% closer to the odorant source than in current day conditions to allow detection (Fig. 3). This would increase the risk of predation or decrease the ability to find food, resulting in a direct impact on survival. This is consistent with the observation that coral reef fish

raised in elevated CO_2 show a 9-fold increase in mortality in the wild[4]. The activity of sea bass exposed to elevated CO_2 was significantly reduced (Fig. 1) likely resulting 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 global gene expression in the olfactory system

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 to explain how elevated CO_2 affects fish behavior

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.

The mechanism of altered neurotransmitter function previously hypothesized to explain the impairments of sensory-induced behaviors observed in coral reef fish is limited to alterations at the level of the brain[43]. It proposes that extracellular acid-base regulatory changes that fish undergo in response to exposure to elevated CO_2 lead to changes in gradients for HCO_3^- and Cl^- ions across neuronal cell membranes in the brain. In turn these changes are suggested to interfere with the normal functioning of the gamma-aminobutyric acid A ($GABA_A$) receptor, causing increased excitation rather than inhibition of the nervous system and the observed downstream behavioral impairments[18, 43]. However, not all fish are good acid-base regulators, and some do not regulate extracellular pH at all when facing elevated CO_2 environments[44]. The mechanism proposed here is independent of any changes in blood acid-base chemistry but is instead dependent on the external (seawater) changes in CO_2/H^+. This raises the possibility that all fish species exposed to elevated CO_2 are potentially susceptible to the direct impairment of peripheral olfactory sensitivity proposed here, whereas the central brain impairment of sensory behavior will principally be relevant to species that are good acid-base regulators.

An apparent discrepancy is that in some studies behavioral abnormalities previously shown for fish exposed to ocean acidification are not evident for the first 24 hours of exposure[4]. However, these previous observations are based on fish exposed to strong odors, probably well above the threshold of detection[3, 4, 43]. Thus, even a 50% reduction in the olfactory sensitivity at these high odorant concentrations would not prevent fish from smelling these strong predator odors, giving rise to some

form of behavioral response under elevated CO_2 conditions. By contrast, the peripheral mechanism proposed here would impair olfaction following any duration of 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. Ultimately, it is becoming clear that an elevated CO_2 environment has the potential for major negative impacts on olfactory-mediated behavior of fish across a wide range of habitats and latitudes. This highlights the potential for ecologically significant population-level impacts on fishes, and perhaps other marine fauna, 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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Figures

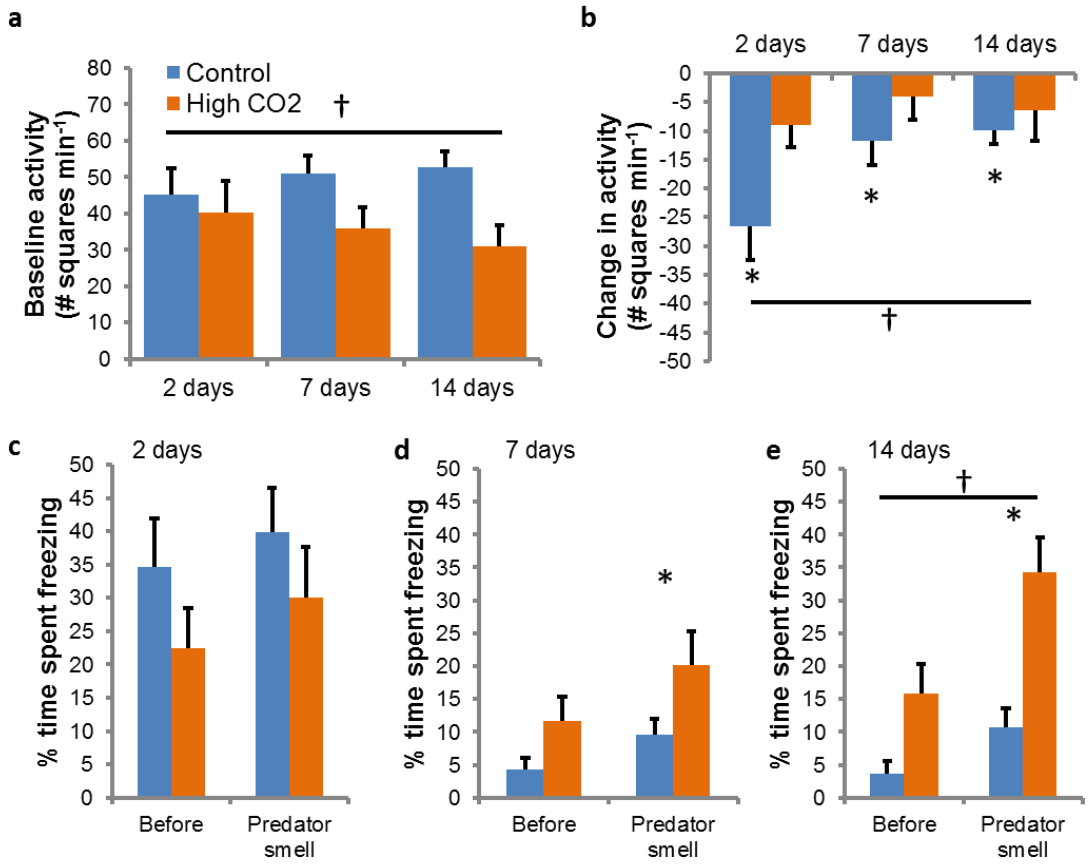

Figure 1. Behavior responses of European seabass (*Dicentrarchus labrax*) to a 5 min exposure to a predator odor (monkfish bile) after exposure to control (~420 µatm) or elevated CO_2 (~950 µatm) for 2, 7, and 14 days. a, baseline activity after 2, 7, and 14 days exposure to control and elevated CO_2. **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 ($p < 0.05$).

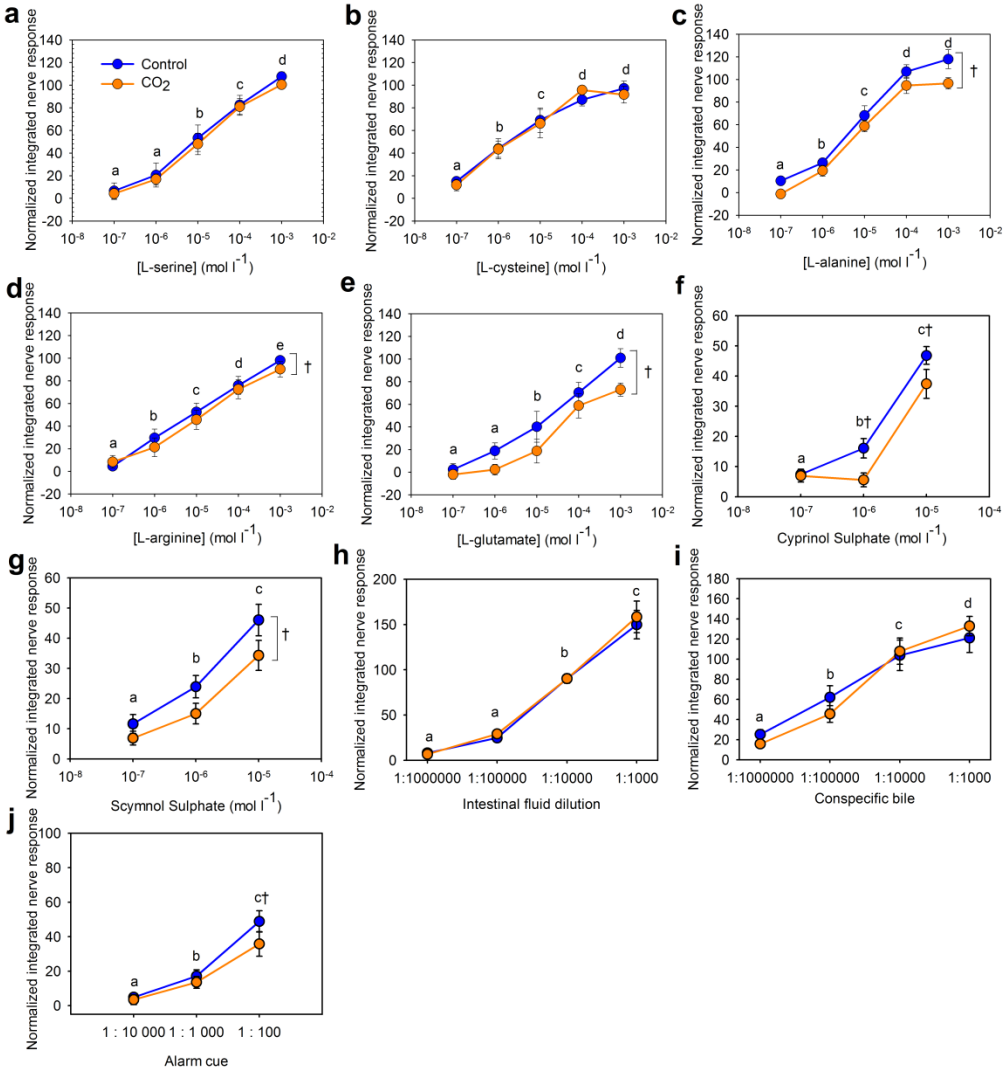

Figure 2. Elevated CO_2/H^+ decreases the olfactory sensitivity of European sea bass to amino acids, bile acids and body fluids. **a**, L-serine (N=6). **b**, L-cysteine (N=6). **c**, L-alanine (N=8). **d**, L-arginine (N=6). **e**, L-glutamate (N=6). **f**, cyprinol sulphate (N=10). **g**, scymnol sulphate (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 Information for raw traces of these responses.

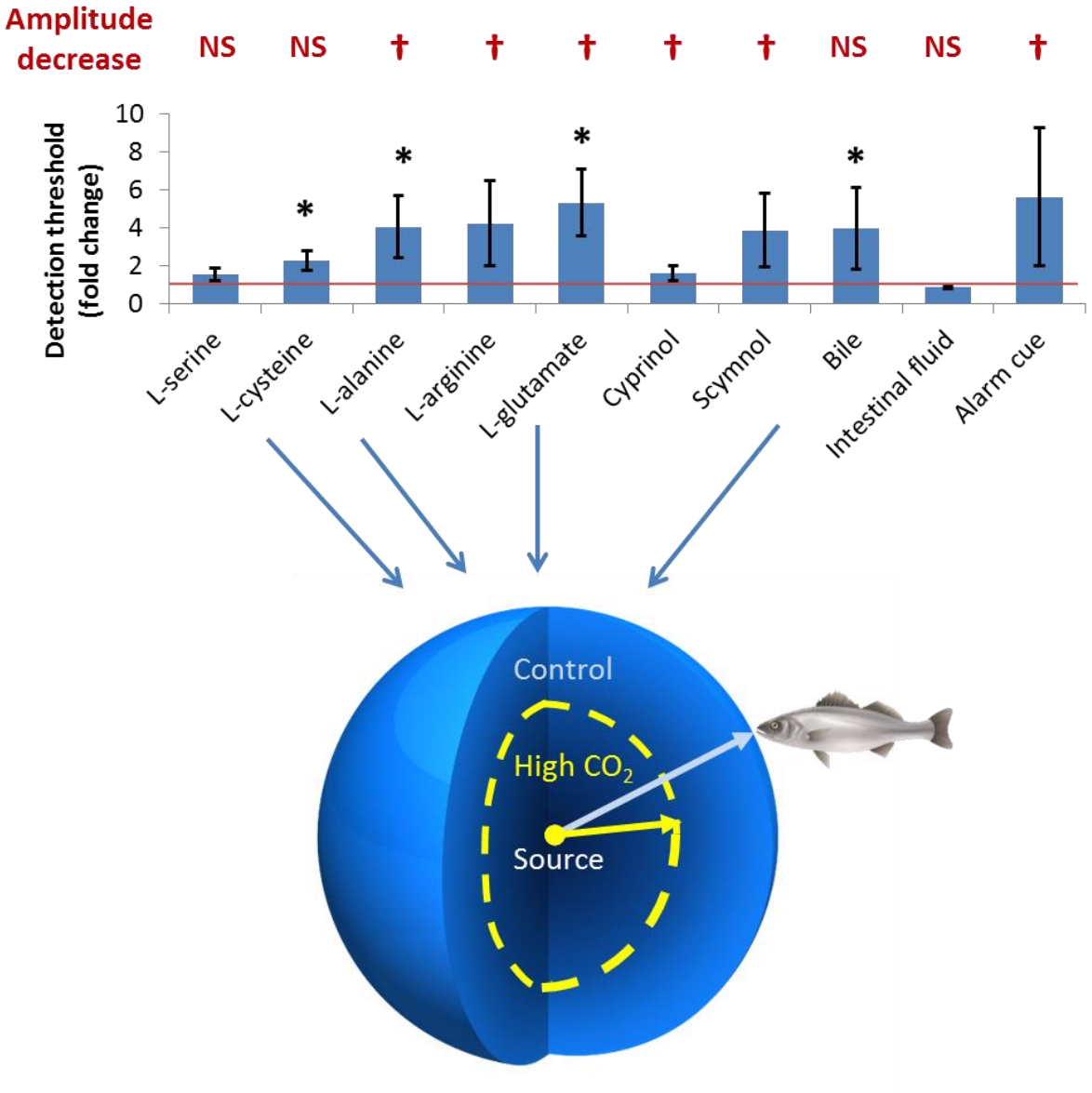

Figure 3. Acute exposure of European seabass to elevated CO_2 (~1000 µatm) decreases the amplitude of the olfactory response and increases the detection threshold of several odorants tested by up to 5 fold. Elevated CO_2 reduces the active space (represented by the blue sphere) of an odor by up to 80% (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 Supplementary materials 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.

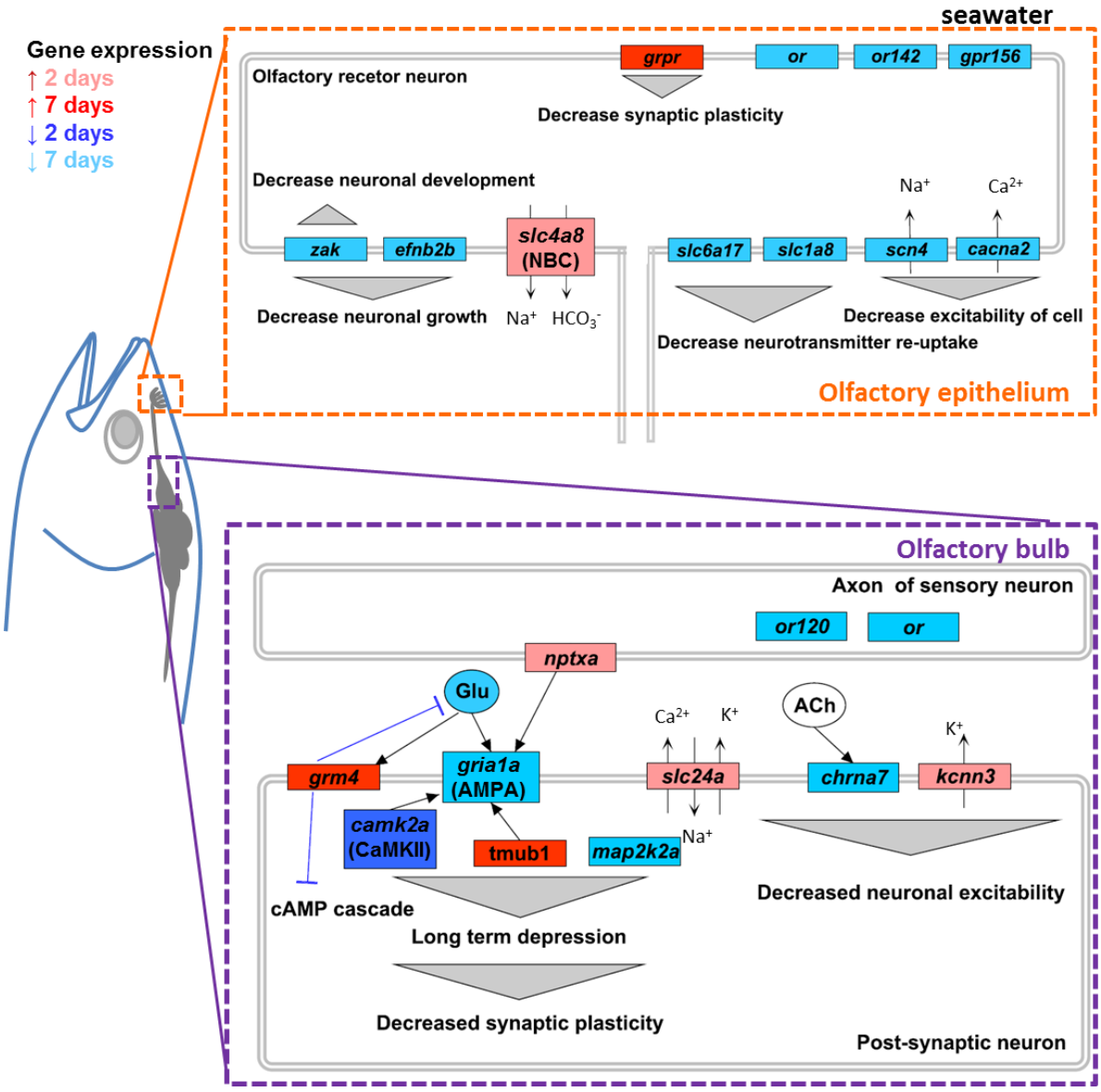

Figure 4. Differential regulation of genes from the olfactory epithelium and olfactory lobe of European sea bass exposed to control and high CO_2 for 2 and 7 days. Genes involved in neuronal growth (*efrn2b*) and development (*zak*) were significantly down-regulated in the olfactory epithelium. Additionally 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.

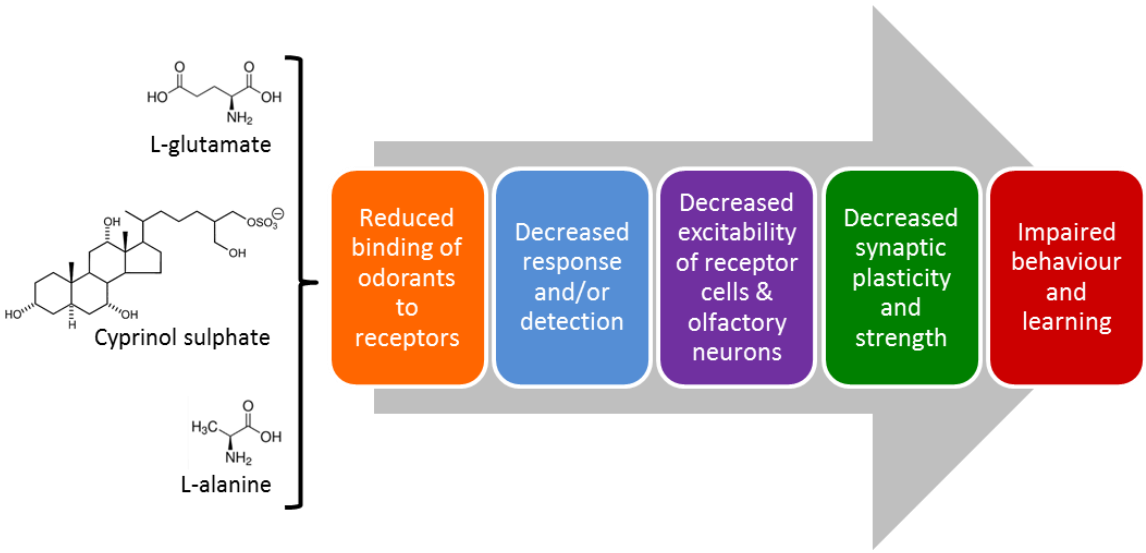

Figure 5. Proposed mechanism of action of CO_2-induced ocean acidification on fish behavior via the olfactory pathway. Ocean acidification has an acute effect on the binding of odorants to their receptors, decreasing both detection threshold and amplitude of the response. Long term exposure to high CO_2 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.