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

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1 **Survival of marine fishes exposed to elevated near-future CO₂ levels is**
2 **threatened by their altered responses to sensory cues. Here we demonstrate a**
3 **novel physiological and molecular mechanism based in the olfactory system**
4 **which helps explain altered behavior under elevated CO₂. We combine**
5 **electrophysiology and high throughput sequencing with behavioral**
6 **experiments to investigate how elevated CO₂ affects the olfactory system of**
7 **European sea bass (*Dicentrarchus labrax*), an economically important species.**
8 **Under elevated CO₂ (~1000 μatm) fish need to be up to 42% closer to an odor**
9 **source for detection, compared with current CO₂ levels (~400 μatm),**
10 **decreasing their chances of detecting food or predators. These findings**
11 **correlated with a suppression in the transcription of genes involved in**
12 **synaptic strength, cell excitability, and wiring of the olfactory system in**
13 **response to sustained exposure to elevated CO₂. Our results contrast with, but**
14 **complement, the previously proposed mechanism of impaired**
15 **neurotransmitter (γ-aminobutyric acid) function, and demonstrate that both the**
16 **olfactory system and central brain function are compromised by elevated CO₂**
17 **in the oceans, with potentially major negative impacts on fish globally.**

18 Fish rely heavily on their olfaction for finding food^{1,2}, recognizing conspecifics and
19 predators^{1,3,4}, for the perception of reproductive status⁵ and homing towards
20 suitable habitats^{6,7,8,9}, including spawning grounds and larval settlement¹⁰. The
21 predicted end-of-the-century CO₂ levels (800-1000 μatm) cause ocean acidification
22 (elevated H⁺, reduced pH) and have been shown to have negative effects on the
23 sensory-related behavior and learning of most fish species studied to-date^{3,4,11,12,13}
24 including sharks^{14,15}. Moreover, coral reef fishes exposed to elevated CO₂ levels
25 predicted for the year 2100, show a 9-fold increase in mortality, when returned to the

26 wild as compared with fish exposed to current conditions⁴. This suggests the
27 potential for a major ecologically-relevant impairment of fitness of marine fishes as a
28 consequence of exposure to elevated CO₂.

29 To explain the effects of elevated CO₂ on fish behavior, changes in brain
30 neurotransmitter function have been proposed as the sole mechanism responsible¹¹,
31 ^{16, 17, 18}. To date, there has been no consideration of mechanisms operating outside
32 of the central processing of sensory information. A direct effect of seawater pH or
33 CO₂ on the peripheral olfactory system was dismissed in marine fish¹⁹ without any
34 empirical testing. We have challenged this view and hypothesized that elevated CO₂
35 would directly affect the olfactory system of fish, given that the olfactory epithelium is
36 in intimate contact with sea water. We propose a novel physiological mechanism by
37 which ocean acidification can alter fish behavior and learning directly via the
38 olfactory system, and used the European sea bass as a model to test this
39 hypothesis.

40 **Results**

41 Firstly, we confirmed that the behavior of European sea bass was affected by
42 elevated CO₂ levels as demonstrated in other fish species. Juvenile sea bass were
43 exposed to current (~430 µatm; control) and predicted end of the century levels of
44 CO₂ (~1000 µatm; elevated CO₂) and their behavioral responses to a likely predator
45 odor²⁰ (bile from monkfish, *Lophius piscatorius*; dilution 1:1,000,000) was quantified.
46 Our data demonstrated that sea bass exposed to elevated CO₂ reduced their
47 baseline activity (swimming) by up to 40% compared with control fish (p=0.008, Fig
48 1a). This difference was independent of the duration of exposure (p=0.30).
49 Furthermore, control sea bass reduced their activity by 50% in the presence of the
50 predator odor, whereas sea bass exposed to elevated CO₂ reduced their activity by

51 only 20-27% ($P=0.009$, Fig. 1b). Both control and elevated CO_2 exposed fish
52 displayed freezing behavior (not moving for more than 5 seconds at a time) after 2
53 and 7 days of exposure (Fig. 1a,b). However, at 14 days, fish exposed to elevated
54 CO_2 spent significantly more time freezing ($p=0.043$) before and during exposure to
55 a predator cue (Fig. 1e).

56 Having established that end-of-century levels of CO_2 in the water result in
57 pronounced alterations of behavior, we tested if exposure to elevated CO_2 at the
58 olfactory epithelium alone was sufficient to reduce the detection of odorants. We
59 used electrophysiological recordings from peripheral sensory neurons of the
60 olfactory system, allowing us to isolate peripheral olfactory responses from central
61 brain processes. We measured changes in the activity of the olfactory nerve whilst
62 exposing the olfactory epithelium to sea water containing ten different olfactory
63 stimuli dissolved in either control or elevated CO_2 seawater, while fish were
64 maintained under control CO_2 levels. We tested the olfactory nerve response of sea
65 bass to a wide range of odorants: amino acids (L-cysteine, L-serine, L-alanine, L-
66 arginine and L-glutamate), as odorants principally mediating food detection²¹; bile
67 acids involved in chemically-mediated interactions between conspecifics and other
68 teleost species (cyprinol sulphate) and potentially predatory shark species (scymnol
69 sulphate)²²; body fluids (intestinal fluid, bile from conspecifics, and alarm cue), potent
70 chemical signals that can elicit behavioral responses vital for escape
71 from/awareness of predators or recognition of conspecifics^{23, 24, 25} (see
72 Supplementary materials for a full description of the methods). The amplitude of the
73 response indicates the change in magnitude of the nerve activity in response to an
74 odorant, and the detection threshold is defined as the concentration of odorant that
75 produced a detectable response (above baseline). Overall, elevated CO_2 reduced

76 the amplitude of the response for 6 out of the 10 odorants compared to control (Fig.
77 2), and increased the detection threshold (i.e. reduced sensitivity) in 4 out of the 10
78 odorants (Fig. 3), but had no effect on the remainder (i.e. elevated CO₂ did not
79 increase amplitude or sensitivity of the response to any of these odorants). Under
80 elevated CO₂ the responses to L-alanine, L-arginine and L-glutamate, cyprinol
81 sulphate, scymnol sulphate and alarm cue were up to 46% lower than those of
82 controls (n=6-11 per odorant per treatment, p=0.027, p=0.040, p=0.028, p=0.011,
83 p=0.012, p=0.018 respectively, Fig. 2). The thresholds of detection for L-cysteine
84 (p=0.049), L-alanine (p=0.029) and L-glutamate (p=0.0047), and conspecific bile
85 (p=0.029) were 2 to 5 fold higher in elevated CO₂ (Fig. 3). Therefore, under elevated
86 CO₂ these odorants would need to be present in the water at concentrations up to 5
87 times greater than in current CO₂ conditions in order to be detected by sea bass.

88 The active space of a chemical is defined as the largest volume of water a fish
89 can occupy that still contains a concentration of odorant at or above the olfactory
90 threshold for detection^{25, 26}. This is a useful parameter to help estimate how much
91 closer a fish would need to be (on average) to detect an odor-source under these
92 elevated CO₂ conditions. We assumed a homogeneous distribution of the odorant in
93 the water. Although this assumption may be simplistic for most natural environments
94 (due to the constant movement of water associated with currents, tidal movements,
95 etc.), it allows for a good estimate of the average change for most circumstances.
96 The largest reduction in threshold of detection (5 fold) was for glutamate (Fig. 3). We
97 calculated that the active space (i.e. a 3 dimensional volume) for glutamate detection
98 would be reduced by 80%, which would translate into fish having to be 42% closer to
99 the odorant source before detection occurred (i.e. based on the one dimensional

100 radius of the three dimensional active space sphere; Fig. 3 - see Supplementary
101 Information for calculation details and assumptions).

102 Lastly, RNA sequencing was used to elucidate the molecular mechanisms
103 underpinning the negative effects of elevated CO₂ on sea bass olfaction. Sea bass
104 were exposed for 2 and 7 days to either control (~450 µatm) or elevated CO₂ (~1000
105 µatm). Global gene transcription was measured in tissue samples taken from the
106 olfactory epithelium (n=6) and the olfactory bulb (n=4) using an Illumina HiSeq 2500
107 platform. *De novo* reference transcriptomes were constructed for each tissue using
108 the Trinity pipeline²⁷ (see Supplementary Information for method description).
109 Transcript abundances were calculated using RSEM²⁸ and differences in gene
110 transcription were determined using EdgeR²⁹ and a selection of scripts provided by
111 Trinity.

112 Calcium/calmodulin-dependent protein kinase II beta 2 (CAMKII) directly
113 regulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate
114 receptors, known to be involved in synaptic plasticity³⁰. In the olfactory bulb, fish
115 exposed to elevated CO₂ for 2 days showed a significant down-regulation of
116 *camk2ga* (gene encoding CAMKII) and *nptxr* (encoding for a protein involved in
117 synaptic plasticity and the clustering of AMPA receptors). This was followed by the
118 downregulation of *gria1b* (homologous to a gene encoding AMPA glutamate
119 receptors), downregulation of *map2k2a* (gene encoding mitogen activated protein
120 kinase kinase involved in olfactory learning in the olfactory bulb in rats³¹), and the
121 upregulation of *tmub1* (gene encoding a protein involved in the regulation of AMPA
122 receptors at the cell surface) at 7 days. Additionally, genes involved in
123 neurotransmitter re-uptake (*slc6a17* and *slc1a8*) including glutamate were
124 downregulated in the olfactory epithelium in fish exposed to elevated CO₂ for 7 days.

125 In the olfactory epithelium there was an upregulation of *slc4a8* (gene encoding the
126 sodium/bicarbonate cotransporter) at 2 days, similar to that observed for long-term
127 adjustments of bicarbonate (HCO_3^-) transport in the gills of marine fish in response to
128 elevated CO_2 ³². Four chemosensory G-protein coupled receptor gene families have
129 been identified in teleosts: main olfactory receptors (ORs), trace amine-associated
130 receptors (TAARs), and vomeronasal receptors 1 and 2 (VR1 and VR2)³³ and all of
131 these were well represented in the olfactory epithelium transcriptome
132 (Supplementary Information). In the olfactory epithelium two OR genes (*or* and
133 *or142*) were significantly down-regulated after 7 days of exposure; however, no OR
134 genes were up-regulated. Interestingly, in the olfactory bulb two additional OR genes
135 (*or* and *or120*), likely expressed in the axons of sensory neurons reaching the
136 olfactory bulb, were also downregulated in fish exposed to elevated CO_2 for 7 days.
137 In mammals, the presence of odorant receptor mRNA in the axons of sensory
138 neurons is well established, and these mRNAs are likely involved in the wiring of the
139 olfactory system³⁴. These results suggest that fish exposed to elevated CO_2 did not
140 activate compensatory molecular mechanisms to adjust for the loss of olfactory
141 sensitivity measured using nerve recording, and that the wiring of the olfactory
142 system might be affected by ocean acidification.

143 Genes involved in excitatory neurotransmission such as the nicotinic
144 acetylcholine receptor (*chrna7*) and glutamate receptor (*gria1b*) were down-
145 regulated in the olfactory bulb of fish exposed to elevated CO_2 levels for 7 days,
146 while a gene involved in decreasing neuronal excitability (calcium-activated
147 potassium channel, *kcnn3*) was up-regulated in the same tissue after 2 days of
148 exposure. In the olfactory epithelium, fish exposed to elevated CO_2 for 7 days
149 showed down-regulation of excitatory neurotransmission (*scn4ab*, *cacna2d*), and

150 neuronal growth and development (*zak* and *efnb2b*). These results indicate
151 mechanisms for decreased excitability of neurons in the olfactory epithelium and
152 bulb at both time points, and thus, a decrease in olfactory information being
153 transmitted to higher brain centers.

154 **Discussion**

155 **Elevated CO₂ affects the behavior of sea bass**

156 Adult sea bass spawn offshore and the newly-hatched larvae must navigate back to
157 the safety of coastal nursery habitats using their olfactory senses to home in on
158 these sites and to avoid predators³⁵, and this life history strategy is shared by many
159 fish species. This is the most vulnerable stage in the life cycle of sea bass being
160 associated with high mortality from predation³⁵. Here, we report that juvenile sea
161 bass exposed to levels of CO₂ predicted for the end of the century demonstrate
162 impaired behavior in response to a predator cue (Fig. 1). This response is similar to
163 those reported for other fish species studied to date and, importantly, an increased
164 effect was observed with exposure time^{3, 4, 11, 12}. However, in sea bass the baseline
165 activity was lower after exposure to elevated CO₂ (Fig. 1), which is unlike most fish
166 species studied to date (generally higher activity was reported after exposure to
167 elevated CO₂)^{36, 37}. Although a recent study found no effects of elevated CO₂ on the
168 routine swimming behavior of early juvenile sea bass³⁸, this could have been
169 influenced by much higher control CO₂ conditions (585 μ atm vs 430 μ atm in our
170 study). This higher level of CO₂ (585 μ atm) is predicted to be reached during the mid
171 21st century, and behavioral impairments were reported in some species following
172 exposure to this level of CO₂³⁹. In our study, juvenile sea bass exposed to elevated
173 CO₂ also spent more time freezing compared to those exposed to control CO₂ levels;

174 this is consistent with previous findings showing that rockfish exposed to elevated
175 CO₂ had elevated levels of anxiety compared to control fish¹¹.

176 **Elevated CO₂ has an acute effect on the olfactory sensitivity of sea bass**

177 The decreased behavioral response to the predator odor was accompanied
178 by a decrease in olfactory sensitivity (either via response amplitude or detection
179 threshold) to 8 out of 10 odorants tested, indicating that olfaction is generally impaired
180 in sea bass exposed to elevated CO₂. The sensitivity of some odorants was more
181 affected than that of others, suggesting that the quality of the perceived odor might
182 be altered in fish exposed to elevated CO₂. This may help to explain the
183 inappropriate (rather than simply inhibited) behavioural responses to complex
184 predator and home-reef odours previously described in the literature^{3, 4}. Furthermore,
185 our results are consistent with studies in freshwater pink salmon (*Oncorhynchus*
186 *gorbuscha*) and marine shore crabs (*Carcinus maenas*) exposed to elevated CO₂
187 showing that olfactory sensitivity is impaired, even after prolonged exposure to
188 elevated CO₂, and can be restored within two hours or less of return to control
189 conditions^{40, 41}, indicating that this effect persists during chronic exposure to elevated
190 CO₂, but is readily reversible. In freshwater fish and marine crabs exposed to
191 elevated CO₂/low pH the loss of response to some odorants, including alarm cue,
192 has been attributed to structural and functional changes of the chemical cues
193 themselves^{19, 41}. This is also a potential explanation for how elevated CO₂ affects
194 odorant-receptor binding in the current study.

195 We estimate that under elevated CO₂ sea bass must be up to 42% closer to
196 the odorant source than in current day conditions to allow detection (Fig. 3). This
197 would increase the risk of predation or decrease the ability to find food, resulting in a
198 direct impact on survival. This is consistent with the observation that coral reef fish

199 raised in elevated CO₂ show a 9-fold increase in mortality in the wild⁴. The activity of
200 sea bass exposed to elevated CO₂ was significantly reduced (Fig. 1) likely resulting
201 in reduced energetic costs. Importantly, this reduced activity would also further
202 reduce their chances of encountering odors in elevated CO₂ conditions. Therefore, if
203 these changes in detection thresholds persist during longer term exposure to
204 elevated CO₂ (as found in freshwater salmon⁴⁰), these could have important
205 ecological consequences at the population level, affecting communication with
206 conspecifics, prey detection and, particularly, predator avoidance.

207 **Elevated CO₂ affects global gene expression in the olfactory system**

208 Only one recent study has investigated the effect of elevated CO₂ on global
209 gene expression patterns in the brain of fish, and found that in juvenile spiny
210 damselfish (*Acanthochromis polyacanthus*) genes associated with brain glucose,
211 serine and glycine metabolism were differentially expressed in fish exposed to
212 elevated CO₂⁴². Interestingly, in our study both electrophysiology and RNA-Seq
213 results indicated that the response to glutamate was most affected by elevated CO₂.
214 We show that in fish exposed to elevated CO₂ genes encoding CAMKII, MAPK
215 kinase and AMPA glutamate receptors were downregulated, and genes associate
216 with AMPA receptor cycling (*tmub1* and *nptx2*) were upregulated. These genes are
217 involved in long term depression (a long-lasting decrease in synaptic strength), a
218 process associated with a decline in learning and memory in higher brain centres³⁰.
219 The expression of genes involved in maintaining neuronal excitability (*chrna7*,
220 *gria1b*, *scn4ab*, *cacna2d*) also decreased in fish exposed to elevated CO₂. A
221 decrease in synaptic plasticity in the olfactory bulb and a decrease in neuronal
222 excitability in the olfactory system suggest that less olfactory information was being
223 sent to higher brain centers. Additionally, OR genes in the olfactory bulb were

224 downregulated in fish exposed to elevated CO₂. These genes have been shown to
225 be involved in the patterning of the olfactory bulb in mammals, particularly during the
226 development of the olfactory system³⁴. Interestingly, these findings are consistent
227 with impaired learning in responding to a predator odor in larval damselfish
228 (*Pomacentrus amboinensis*) exposed to elevated CO₂¹³, perhaps due to reduced
229 olfactory information reaching higher brain centers, compromising learning and
230 memory formation.

231 **Novel physiological mechanism to explain how elevated CO₂ affects fish**
232 **behavior**

233 We propose a novel mechanism based in the olfactory system to explain how
234 elevated CO₂ alters the behavior of fish (Fig. 5). First, we show that elevated CO₂
235 can have a direct effect on the sensitivity of olfactory reception to various odorants in
236 sea bass, likely by reduced affinity of odorant-receptor binding in the olfactory
237 epithelium. Our electrophysiology data show that fewer impulses are sent to the
238 olfactory bulb in response to most odorants, regardless of concentration. This would
239 result in a decrease in the activity of olfactory bulb synapses, detected by a change
240 in the timing and the frequency of calcium cycling in these neurons, a process that
241 can lead to a decrease in synaptic plasticity³⁰. Indeed, gene expression results show
242 that sea bass exposed to elevated CO₂ downregulate genes involved in synaptic
243 plasticity and maintaining the excitability of both peripheral olfactory receptor
244 neurons and central olfactory bulb neurons, supporting our hypothesis. Therefore,
245 we propose that under future levels of elevated CO₂ fish may sense less information
246 through their olfactory receptors, and this would be compounded by less peripheral
247 olfactory information being transmitted to higher brain centers. Additionally, we also
248 found decreases in the expression of genes involved in in the wiring of the olfactory

249 system, an important developmental process for juvenile fish. These physiological
250 and molecular changes are consistent with the altered behavior observed in this
251 study and others and have strong implications for fitness in the wild.

252 The mechanism of altered neurotransmitter function previously hypothesized to
253 explain the impairments of sensory-induced behaviors observed in coral reef fish is
254 limited to alterations at the level of the brain⁴³. It proposes that extracellular acid-
255 base regulatory changes that fish undergo in response to exposure to elevated CO₂
256 lead to changes in gradients for HCO₃⁻ and Cl⁻ ions across neuronal cell membranes
257 in the brain. In turn these changes are suggested to interfere with the normal
258 functioning of the gamma-aminobutyric acid A (GABA_A) receptor, causing increased
259 excitation rather than inhibition of the nervous system and the observed downstream
260 behavioral impairments^{18, 43}. However, not all fish are good acid-base regulators, and
261 some do not regulate extracellular pH at all when facing elevated CO₂
262 environments⁴⁴. The mechanism proposed here is independent of any changes in
263 blood acid-base chemistry but is instead dependent on the external (seawater)
264 changes in CO₂/H⁺. This raises the possibility that all fish species exposed to
265 elevated CO₂ are potentially susceptible to the direct impairment of peripheral
266 olfactory sensitivity proposed here, whereas the central brain impairment of sensory
267 behavior will principally be relevant to species that are good acid-base regulators.

268 An apparent discrepancy is that in some studies behavioral abnormalities
269 previously shown for fish exposed to ocean acidification are not evident for the first
270 24 hours of exposure⁴. However, these previous observations are based on fish
271 exposed to strong odors, probably well above the threshold of detection^{3, 4, 43}. Thus,
272 even a 50% reduction in the olfactory sensitivity at these high odorant concentrations
273 would not prevent fish from smelling these strong predator odors, giving rise to some

274 form of behavioral response under elevated CO₂ conditions. By contrast, the
275 peripheral mechanism proposed here would impair olfaction following any duration of
276 elevated CO₂ conditions, particularly when odorants are close to their detection
277 threshold, a more realistic scenario in a natural environment. Secondly, the
278 behavioral responses documented previously are downstream of the central brain
279 GABA-regulated processes that should only be impaired secondary to acid-base
280 regulation and changes in blood chemistry⁴⁵. It is also important to recognize that the
281 two models (the one proposed here based in the olfactory system and the previously
282 proposed impairment of GABA receptor function) are not mutually exclusive. Indeed,
283 it seems likely that they would operate together during exposures lasting longer than
284 24 h, in particular for acid-base regulators, impacting sensory behavior through two
285 distinct physiological mechanisms and ultimately impairing fitness.

286 **Conclusions**

287 Recent studies, including ours, indicate that behavioral responses persist, or
288 become more pronounced, with prolonged experimental exposure to elevated CO₂⁴,
289 ¹² and in fish that live in naturally high CO₂ environments (near CO₂ seeps)⁴⁶.
290 Additionally, it is not known if the relatively fast change in CO₂ predicted for this
291 century would allow sea bass and other fishes to acclimate or adapt to a high CO₂
292 world, but one generation is apparently not enough to mitigate the effects of elevated
293 CO₂⁴⁷. We propose that the impairment of sensory behavior is induced via not one,
294 but two complementary physiological mechanisms, acting on the olfactory system
295 and on the GABA receptor function in the brain. In essence, fish are impacted at two
296 distinct levels of the sensory-behavioral system, both at the periphery and the central
297 nervous system affecting their behavior. This suggests that complete adaptation may
298 require phenotypic modification at both of these targets of CO₂ exposure. In turn this

299 could either increase selection pressure on this sensory pathway or increase the
300 time required for selection compared to if there was only one target mechanism, and
301 thus complicate predictions about the length of time required for adaptation to occur.
302 Ultimately, it is becoming clear that an elevated CO₂ environment has the potential
303 for major negative impacts on olfactory-mediated behavior of fish across a wide
304 range of habitats and latitudes. This highlights the potential for ecologically
305 significant population-level impacts on fishes, and perhaps other marine fauna,
306 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

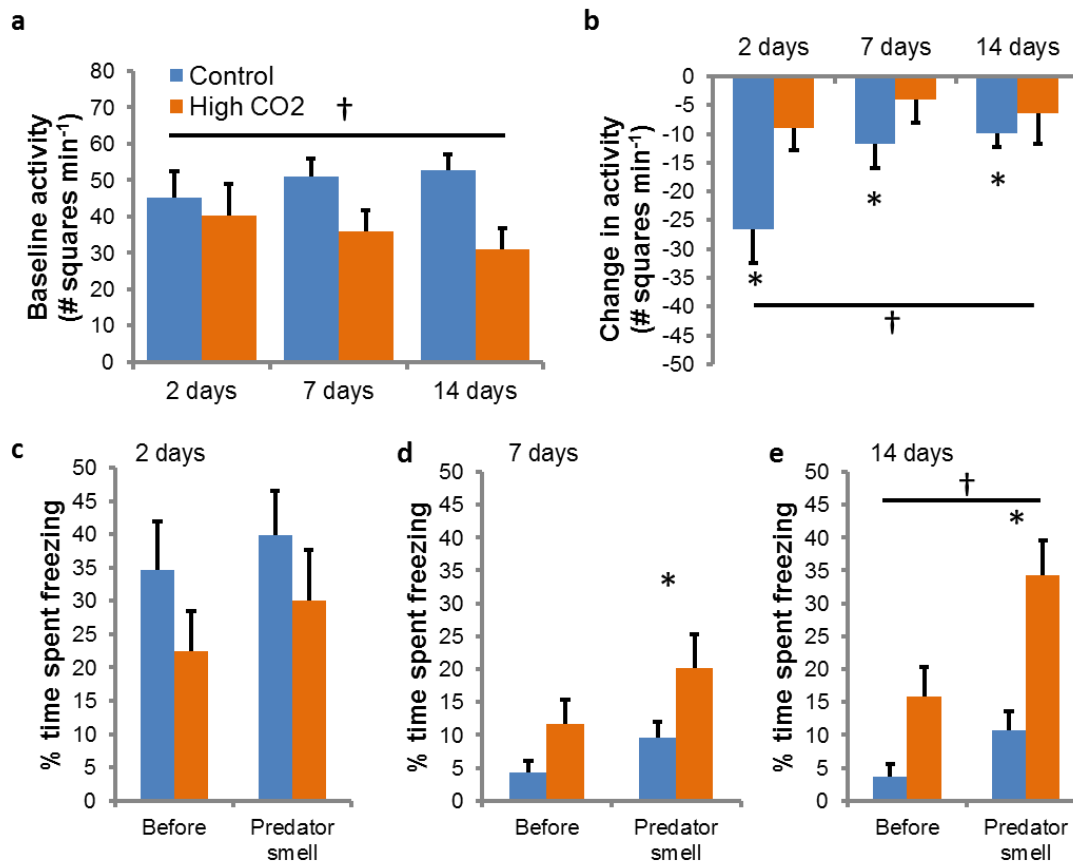


Figure 1. Behavior responses of European seabass (*Dicentrarchus labrax*) to a 5 min exposure to a predator odor (monkfish bile) after exposure to control (~420 μ atm) or elevated CO₂ (~950 μ atm) for 2, 7, and 14 days. **a, baseline activity after 2, 7, and 14 days exposure to control and elevated CO₂. **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 \pm 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 CO₂ treatments ($p < 0.05$).**

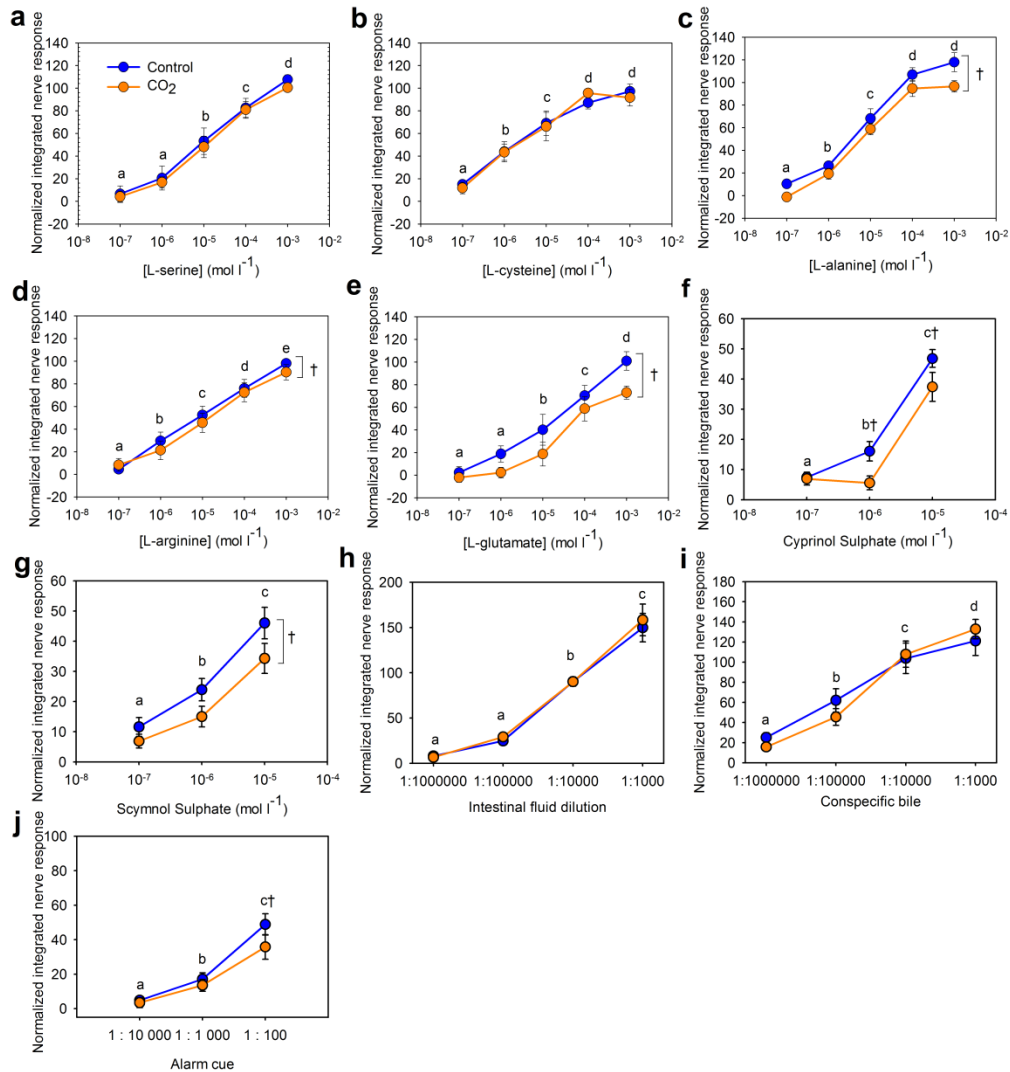


Figure 2. Elevated CO₂/H⁺ decreases the olfactory sensitivity of European sea bass to amino acids, bile acids and body fluids. a, L-serine (N=6). b, L-cysteine (N=6). c, L-alanine (N=8). d, L-arginine (N=6). e, L-glutamate (N=6). f, cyprinol sulphate (N=10). g, scymnol sulphate (N=10). h, intestinal 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 CO₂ (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.

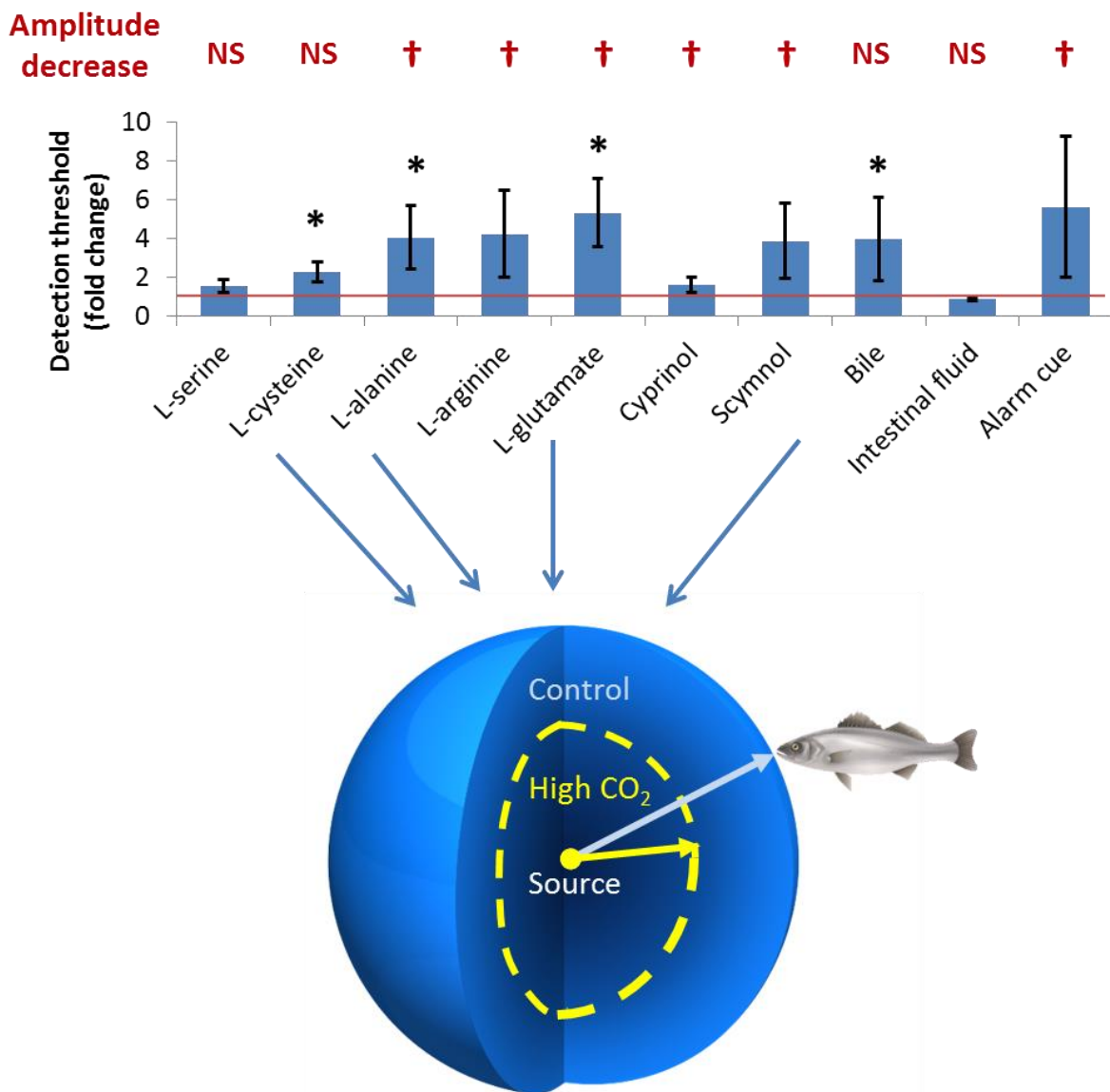


Figure 3. Acute exposure of European seabass to elevated CO₂ (~1000 μatm) decreases the amplitude of the olfactory response and increases the detection threshold of several odorants tested by up to 5 fold. Elevated CO₂ reduces the active space (represented by the blue sphere) of an odor by up to 80% (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.

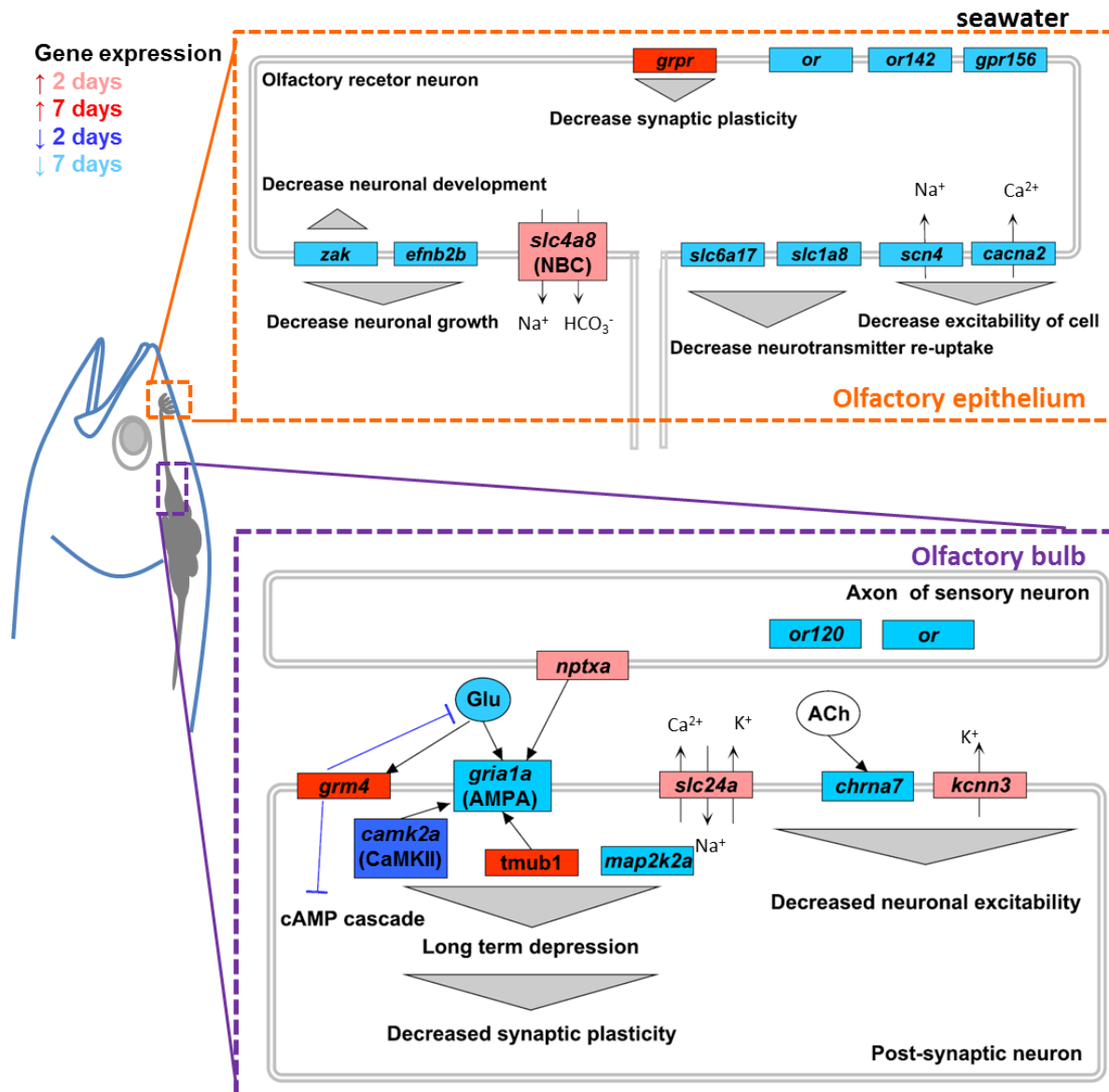


Figure 4. Differential regulation of genes from the olfactory epithelium and olfactory lobe of European sea bass exposed to control and high CO₂ for 2 and 7 days. Genes involved in neuronal growth (*efn2b*) and development (*zak*) were significantly down-regulated in the olfactory epithelium. Additionally 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.

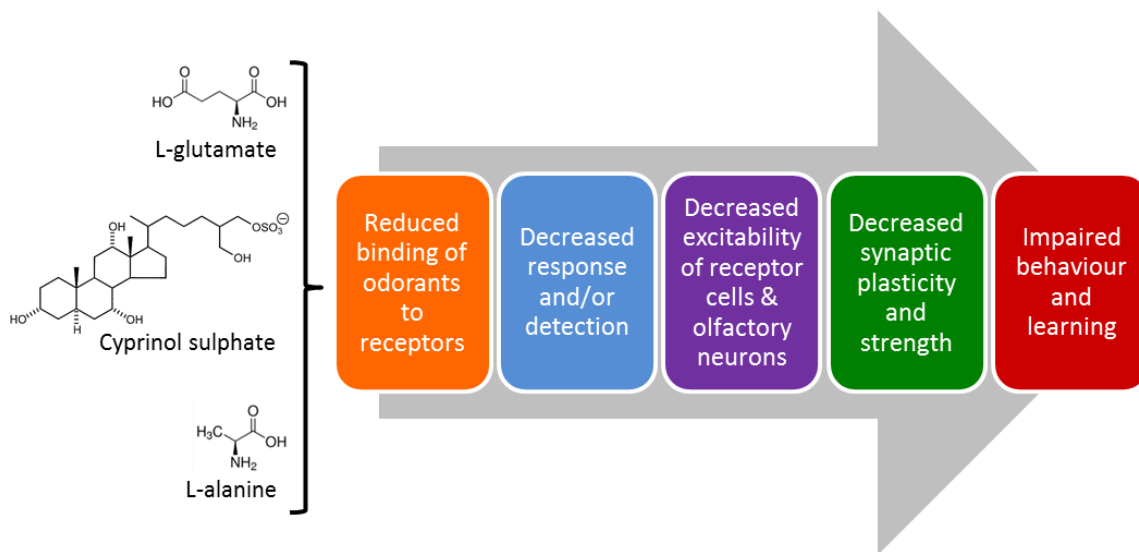


Figure 5. Proposed mechanism of action of CO₂-induced ocean acidification on fish behavior via the olfactory pathway. Ocean acidification has an acute effect on the binding of odorants to their receptors, decreasing both detection threshold and amplitude of the response. Long term exposure to high CO₂ 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.