

# Responses of pink salmon to CO<sub>2</sub>-induced aquatic acidification

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**Ocean acidification negatively affects many marine species and is predicted to cause widespread changes to marine ecosystems. Similarly, freshwater ecosystems may potentially be affected by climate-change-related acidification; however, this has received far less attention. Freshwater fish represent 40% of all fishes, and salmon, which rear and spawn in freshwater, are of immense ecosystem, economical and cultural importance. In this study, we investigate the impacts of CO<sub>2</sub>-induced acidification during the development of pink salmon, in freshwater and following early seawater entry. At this critical and sensitive life stage, we show dose-dependent reductions in growth, yolk-to-tissue conversion and maximal O<sub>2</sub> uptake capacity; as well as significant alterations in olfactory responses, anti-predator behaviour and anxiety under projected future increases in CO<sub>2</sub> levels. These data indicate that future populations of pink salmon may be at risk without mitigation and highlight the need for further studies on the impact of CO<sub>2</sub>-induced acidification on freshwater systems.**

Future predicted increases in CO<sub>2</sub> have been demonstrated to cause a wide range of sublethal effects on a variety of marine fish species. Effects include changes in olfactory responses to predator, prey and substrate cues<sup>1–3</sup>, interference with neurotransmitter function<sup>4,5</sup>, alterations in behavioural lateralization<sup>4,6</sup> and heightened anxiety<sup>5</sup>. Despite the growing body of work on the effects of ocean acidification (OA) on marine systems, less is known about the future patterns and dynamics of CO<sub>2</sub> in freshwater, making it difficult to infer how freshwater ecosystems will respond to climate-change-related acidification. Nevertheless, elevated CO<sub>2</sub> in both marine and freshwater systems is a likely scenario in the future<sup>7</sup>. Although freshwater comprises only 0.8% of the water on the Earth's surface, freshwater ecosystems support almost 40% of all fish species<sup>8</sup>. Therefore, investigating the effects of CO<sub>2</sub>-mediated acidification in freshwater systems may provide important insights into how almost half of the world's fishes and associated communities will respond to climate change.

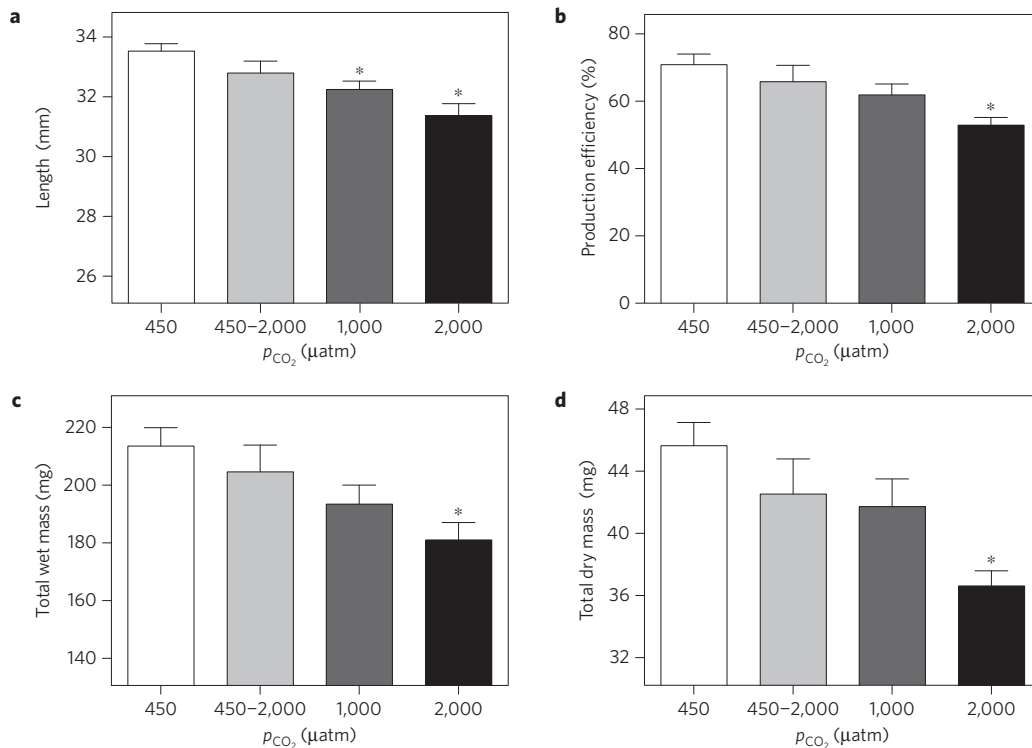
Salmon are a keystone species in many marine, freshwater and terrestrial ecosystems because of their role in supporting food webs<sup>9,10</sup> and, as such, may help link distinct and relatively isolated systems. All salmon initially rear in freshwater but spend the majority of their juvenile and adult lives in seawater, before returning to their freshwater natal streams to spawn<sup>11</sup>. Thus, the freshwater environment is crucial to their life history. In comparison to all other Pacific salmon, pink salmon (*Oncorhynchus gorbuscha*) are the most abundant and widely distributed and, consequently, considered an important indicator of ecosystem health<sup>12</sup>. Unlike most other anadromous salmonids, pink salmon migrate to sea soon after emergence and, at approximately 0.2 g, are by far the smallest at the time of seawater entry<sup>13,14</sup>. As seawater entry is a time of heightened mortality for all species of salmon<sup>15–17</sup>, their small size and high ratio of surface area to volume may make pink salmon especially vulnerable to environmental stressors, including OA.

Here, we show negative effects of CO<sub>2</sub>-induced acidification on the growth, metabolism, olfactory responses and anti-predator behaviour in the early life stages of pink salmon during freshwater development and post-seawater entry using predicted future levels of CO<sub>2</sub>. Unlike other studies, we also incorporated a fluctuating CO<sub>2</sub> treatment to reflect naturally occurring CO<sub>2</sub> fluctuations in freshwater and coastal ecosystems<sup>18–20</sup>.

Two weeks before hatch, eyed embryos were transferred into one of four freshwater treatments (constant 450 μatm (present-day control), constant 1,000 μatm (100-year projection), constant 2,000 μatm, and diurnal fluctuating 450–2,000 μatm) for ten weeks. Growth, routine (RMR) and maximum (MMR) metabolic rate were measured throughout freshwater development and, at the end of ten weeks, their levels of anxiety and olfactory and anti-predator responses to conspecific alarm cues were measured. When fish reached yolk sac absorption (week 10 post-CO<sub>2</sub> exposure), fish reared in freshwater at constant 450 μatm CO<sub>2</sub> were transferred to one of three different seawater treatments of 450, 1,600 (future coastal conditions) and a diurnal 450–1,600 μatm CO<sub>2</sub> treatment. A subset of fish reared at constant 2,000 μatm CO<sub>2</sub> in freshwater were also transferred into 1,600 μatm seawater (Supplementary Information). Growth and metabolic rates were measured over the subsequent two weeks post-seawater transfer.

During freshwater development, there was a significant negative effect of CO<sub>2</sub> on fork length, production efficiencies, total wet mass and total tissue mass (yolk removed) and no significant effect of CO<sub>2</sub> on RMR and MMR (Supplementary Information). At yolk sac absorption (week 10), total wet mass ( $F_3 = 4.0157$ ,  $p = 0.0218$ ), total dry mass ( $F_3 = 4.7993$ ,  $p = 0.0112$ ), production efficiencies ( $F_3 = 4.5412$ ,  $p = 0.0139$ ) and fork lengths ( $F_3 = 8.0345$ ,  $p = 0.0010$ ) were significantly different among groups (see Fig. 1). Production efficiencies (% of yolk converted into tissue) near yolk sac absorption were 25% lower in the high-CO<sub>2</sub> group

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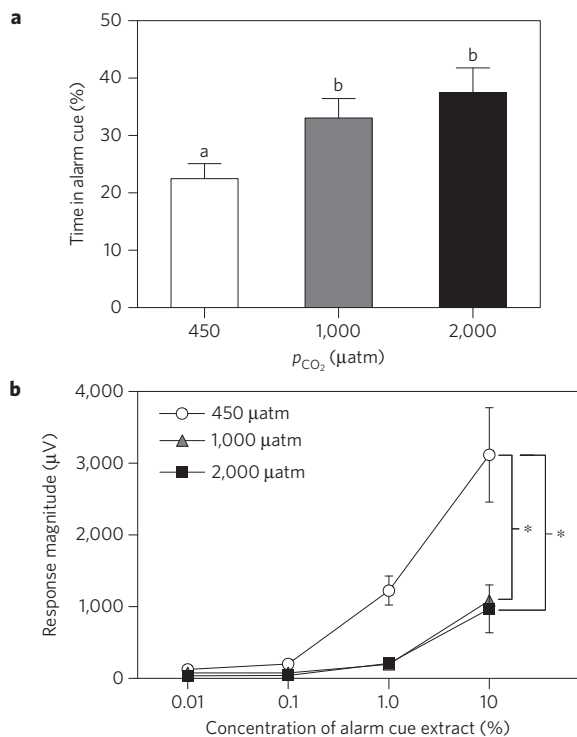
**Figure 1 | Growth measurements in pink salmon (*Oncorhynchus gorbuscha*) at yolk sac absorption (following ten weeks of  $\text{CO}_2$  exposure in freshwater).** **a**, Body lengths. **b**, Production efficiencies (net tissue produced/net yolk consumed  $\times 100$ ). **c**, Total wet mass. **d**, Total dry mass. 450–2,000  $\mu\text{atm}$  represents a diurnal cycle and other tensions are constant throughout. Values are means  $\pm$  s.e.m. ( $n=8$ ). Asterisks indicate a statistically significant difference from the 450  $\mu\text{atm}$  control group ( $p < 0.05$ ).

compared to the control ( $p=0.0064$ ). Similarly, fork lengths were significantly reduced at 1,000 and 2,000  $\mu\text{atm}$  compared to control levels ( $p=0.0292$  and  $p=0.0004$ , respectively).

In freshwater before seawater transfer, we found significant changes in olfactory responses to alarm cues and amino acids, changes in anti-predator responses to alarm cues, and decreased anxiety in pink salmon fry under future predicted levels of  $\text{CO}_2$ .  $\text{CO}_2$  effects on olfactory responses and behaviour seem to be dose dependent, with more pronounced effects occurring at higher  $p_{\text{CO}_2}$ . Elevated  $p_{\text{CO}_2}$  in freshwater had a significant effect on alarm cue response behaviour ( $F_2=5.6512$ ,  $p=0.0072$ , Fig. 2a). All fish had a tendency to avoid alarm cues; however, larvae reared at 1,000 and 2,000  $\mu\text{atm}$  (pH 6.94 and 6.65, respectively) spent significantly more time in water containing alarm cues than larvae reared in control conditions ( $p=0.0358$  and  $p=0.0046$ , respectively). Consistent with our behavioural findings, olfactory epithelial electro-olfactogram (EOG) responses to alarm cues were significantly reduced at elevated  $\text{CO}_2$  levels ( $F_2=13.807$ ,  $p=0.006$ , Fig. 2b) of 1,000 and 2,000  $\mu\text{atm}$  ( $p=0.013$  and  $p=0.010$ , respectively). Similar reductions in EOG responses to various amino acids were also measured in pink salmon under elevated  $\text{CO}_2$  (Fig. 3). EOG responses of 2,000  $\mu\text{atm}$ -reared fish measured in rearing conditions were significantly lower than control fish (cysteine:  $F_8=4.670$ ,  $p=0.002$ ; threonine:  $F_8=2.666$ ,  $p=0.029$ ; glutamate:  $F_8=3.645$ ,  $p=0.007$ ; Fig. 3), but were restored after a 2 h acclimation to control freshwater conditions (cysteine:  $F_4=-5.341$ ,  $p=0.006$ ; threonine:  $F_4=-4.648$ ,  $p=0.010$ ; glutamine:  $F_4=-3.595$ ,  $p=0.023$ ; glutamate:  $F_4=-2.912$ ,  $p=0.044$ ; Fig. 3). Unlike control fish, fish reared at elevated  $p_{\text{CO}_2}$  in freshwater showed a drastic shift in their location preference during our novel approach test (Fig. 4). 2,000  $\mu\text{atm}$ -reared fish spent significantly more time in the centre zone of the arena near the novel object than control fish, which may be an indication of increased boldness ( $z=2.5377$ ,

$p=0.0212$ , Fig. 4a). Control fish also exhibited stronger thigmotaxis (swimming around the walls of a tank), which is a common index of anxiety<sup>21</sup>. Fish reared at 2,000  $\mu\text{atm}$  spent significantly less time in the thigmotaxis zone compared to control fish ( $z=-2.7555$ ,  $p=0.0113$ , Fig. 4b), suggesting that elevated  $\text{CO}_2$  may reduce anxiety in pink salmon fry. Although not statistically significant, fish reared at 1,000  $\mu\text{atm}$  had a tendency for lower anxiety, spending less time in the thigmotaxis zone and more time in the centre than control fish (Fig. 4). To validate the novel approach test as an index of anxiety in pink salmon, we applied gabazine, a drug found to induce anxiety in fish<sup>5</sup>. Gabazine treatment increased the tendency for thigmotaxis in control fish and reversed the effect of  $\text{CO}_2$  on location preference in 2,000  $\mu\text{atm}$ -reared fish (Fig. 4). Overall, gabazine increased anxiety in control fish and completely abolished the anxiety-reducing effects of elevated  $\text{CO}_2$ .

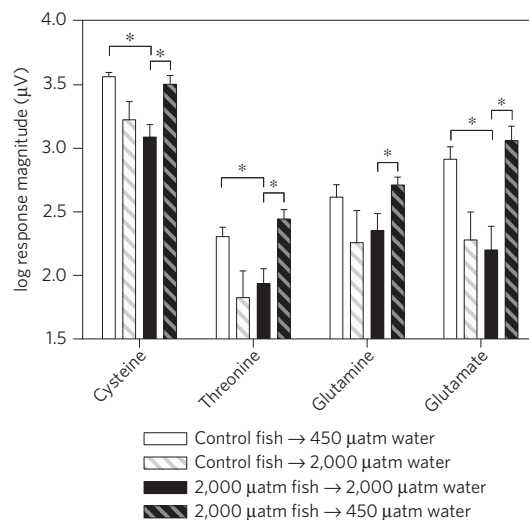
Following seawater transfer, growth and MMR were significantly reduced at elevated  $\text{CO}_2$  levels. Growth rates in seawater were significantly affected by  $\text{CO}_2$  ( $F=6.4692$ ,  $p=0.0031$ , Fig. 5) and were negative in fish transferred into high- $\text{CO}_2$  seawater regardless of their freshwater rearing conditions, whereas control fish continued to grow. At day 14 following seawater transfers, growth rates were significantly lower in fish transferred into high- $\text{CO}_2$  seawater (High FW  $\rightarrow$  High SW and Ctl FW  $\rightarrow$  High SW) than in fish reared in control freshwater and transferred into control seawater (Ctl FW  $\rightarrow$  Ctl SW) ( $p=0.0011$  and  $p=0.0140$ , respectively). There were no significant effects of  $\text{CO}_2$  on RMR post-seawater transfer (Supplementary Information). However, MMR increased significantly following seawater transfer ( $F_{1,77.1}=37.498$ ,  $p < 0.0001$ , Fig. 6), with significant differences among treatments ( $F_{3,5.1}=13.113$ ,  $p=0.0078$ , Fig. 6). Just one week post-seawater transfer, MMR was 27% lower in the High FW  $\rightarrow$  High SW group ( $p=0.0197$ , Fig. 6) and 31% lower in the Ctl FW  $\rightarrow$  High SW ( $p=0.0127$ , Fig. 6) group compared to the Ctl FW  $\rightarrow$  Ctl SW group.



**Figure 2 | Predator avoidance behaviour and olfactory responses of pink salmon reared at different  $p_{\text{CO}_2}$  tensions to conspecific alarm cues in freshwater.** **a**, Time fish spent in water containing the presence of alarm cue ( $0.5 \text{ mg ml}^{-1}$  of skin extract) in the different  $p_{\text{CO}_2}$  tensions that fish were reared in ( $n=15$ ). Letters that differ indicate statistically significant differences among groups. **b**, Electro-olfactogram response magnitude at the olfactory epithelium in response to varying concentrations ( $10^1$ – $10^4$ ) of alarm cue skin extract ( $0.1 \text{ g ml}^{-1}$ ) in the different  $p_{\text{CO}_2}$  tensions that fish were reared in ( $n=3$ ). Asterisks indicate statistically significant differences between indicated groups ( $p < 0.05$ ). Values are means  $\pm$  s.e.m.

### Growth and development in freshwater

Our results indicate that future increases in  $\text{CO}_2$  in freshwater may have substantial negative impacts on the growth, olfactory responses and anti-predator behaviour of pink salmon during early development, which may have large implications on their outward migration success in freshwater and early seawater survival. Near the end of yolk absorption, total wet mass and length were reduced in fish reared at 1,000 and 2,000  $\mu\text{atm}$  compared to those reared in control conditions. Reductions in yolk-to-tissue conversion efficiencies at elevated  $p_{\text{CO}_2}$  may indicate that development at  $p_{\text{CO}_2}$  levels projected by the end of the century may incur a greater energetic cost associated with acid–base regulation. Previous work investigating growth in fish at OA-relevant levels demonstrates varying responses<sup>22–24</sup>, suggesting that OA impacts on growth may be species specific. Early in embryonic development, Japanese medaka, *Oryzias latipes*, show stunted growth under constant elevated  $p_{\text{CO}_2}$ , but this was no longer evident by hatch<sup>22</sup>. In the case of pink salmon, a shift in developmental time does not seem to be occurring. Near yolk absorption, yolk wet mass is not significantly different among the different  $\text{CO}_2$  groups, suggesting that yolk consumption rates were similar (Supplementary Information). Given that gravel emergence in pink salmon occurs at yolk sac absorption, these data indicate that elevated  $\text{CO}_2$  in freshwater would probably lead to comparable times of gravel emergence, but in fish that are smaller. Predation, in most cases, is strongly size-selective<sup>25</sup>; thus, smaller hatchlings may have lower survivorship<sup>26</sup>. This has large implications on the survival of pink salmon not only in freshwater but during early

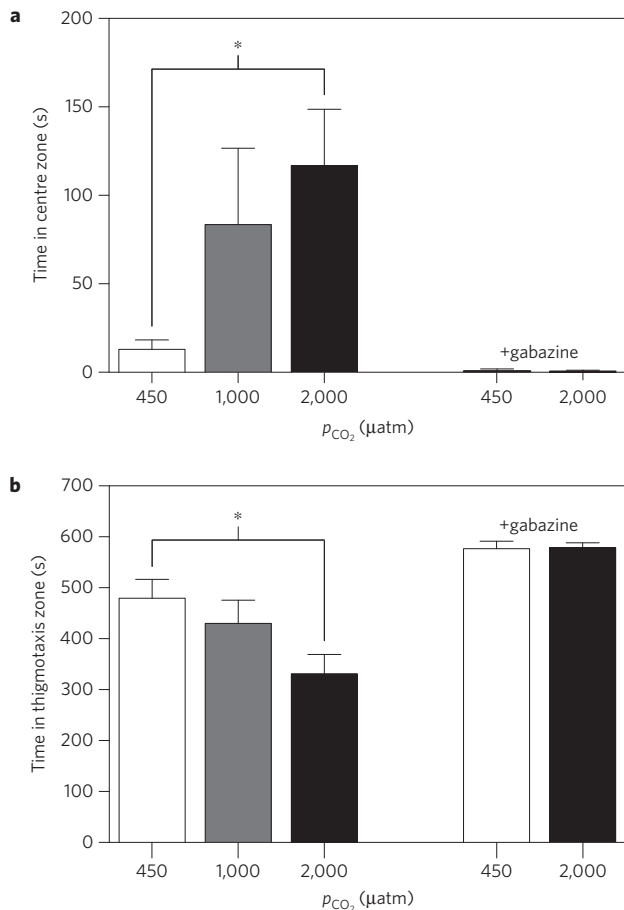


**Figure 3 | Electrophysiological responses at the olfactory epithelium of pink salmon (week 8 of  $\text{CO}_2$  exposure) in response to various amino acids ( $10^{-3} \text{ M}$ ) at different  $p_{\text{CO}_2}$  tensions in freshwater.** Fish reared at 450  $\mu\text{atm}$  or 2,000  $\mu\text{atm}$  in freshwater were tested for their EOG responses to amino acids in their respective water types and then transferred to 2,000  $\mu\text{atm}$  or 450  $\mu\text{atm}$ , respectively for 2 h, after which EOG responses to the same amino acids were measured. Values are means  $\pm$  s.e.m. ( $n=5$ ). Asterisks indicate statistically significant differences between indicated groups ( $p < 0.05$ ).

ocean entry as they make the transition to a more predator-rich marine environment.

### Impaired olfaction, behaviour and homing

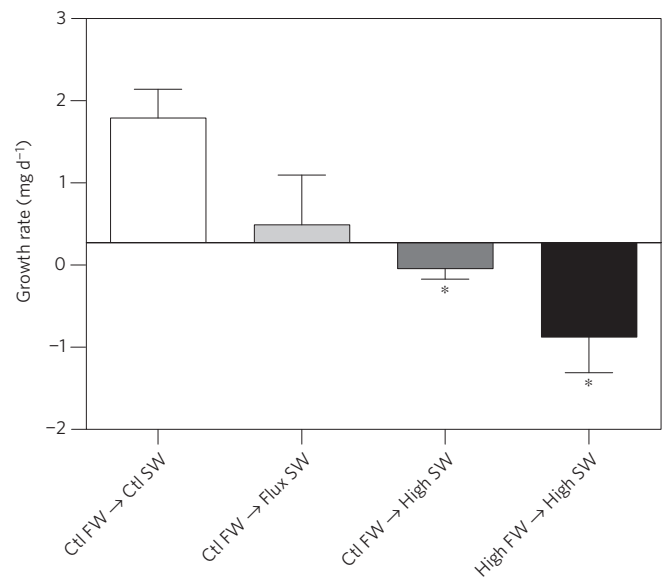
In addition to impaired growth, the diminished capacity for pink salmon fry to detect olfactory cues and elicit predator avoidance behaviour under projected increases in  $\text{CO}_2$  may have negative consequences on survival by altering predator–prey dynamics. Most of the studies to date investigating the effect of acidification on olfactory and behavioural responses to chemical cues have largely been conducted on marine species<sup>1,4,27,28</sup> or in freshwater species at relatively low pH (pH 5.0–6.3), with the use of strong acids ( $\text{H}_2\text{SO}_4$  or  $\text{HCl}$ ) to acidify the environment<sup>29–31</sup>. The latter will not affect internal receptors to the same degree because membrane permeability to charged  $\text{H}^+$ 's is much lower than that of  $\text{CO}_2$ . Compared to pink salmon in freshwater, marine fish also exhibited similar impairment in detecting chemical cues at elevated  $\text{CO}_2$  levels. For example, short-term exposure to high  $\text{CO}_2$  reduced anti-predatory behaviour and increased predation by a common predator in juvenile damselfish, *Pomacentrus sp.*<sup>2,28</sup>. In freshwater, acidification to pH  $< 6.3$  seems to diminish or completely abolish predator avoidance behaviour in various fish species, including other salmonids<sup>29,31,32</sup>. Whereas transgenerational acclimation has been documented to mitigate some of the negative effects of  $\text{CO}_2$  on growth and aerobic scope in reef fish<sup>33</sup>, it had little effect on restoring olfactory responses to alarm cues and behaviour lateralization in juvenile spiny damselfish, *Acanthochromis polyacanthus*<sup>34</sup>. This suggests that genetic adaptation would be required to mitigate the effects of elevated  $\text{CO}_2$  on sensory and cognitive impairment<sup>34</sup>. Consistent with our EOG data, olfactory responses in Atlantic salmon parr (*Salmo salar*) to common odorants were significantly reduced at low freshwater pH (4.5 and 5.5; ref. 35). Below a pH of 6.5 (via  $\text{H}_2\text{SO}_4$  addition), significantly higher concentrations of odorants were needed to produce responses of similar magnitude to controls<sup>35</sup>. Reduced EOG responses to alarm cues and amino acids suggest that elevated  $\text{CO}_2$  may impair olfactory sensitivity to common odorants in pink salmon. The recognition of the



**Figure 4 | Time pink salmon spent in different zones (centre and thigmotaxis zone) in a novel approach test in fish reared and tested in different freshwater  $p_{\text{CO}_2}$  tensions.** **a**, Time fish spent in the centre zone of the arena. **b**, Time fish spent in the thigmotaxis zone (near walls of the arena). Values along the x-axis represent  $p_{\text{CO}_2}$  levels in  $\mu\text{atm}$  that the fish was reared and tested at. In separate fish, gabazine was applied for 30 min before testing in freshwater of the same  $p_{\text{CO}_2}$  tension. Values are means  $\pm$  s.e.m. ( $n=10-15$ ). Asterisks indicate a statistically significant difference from the control group ( $p < 0.05$ ).

amino acid composition of natal streams is thought to play a large role in imprinting and homing migration of salmon<sup>36,37</sup>. If future levels of  $\text{CO}_2$  impair the recognition of amino acids, then homing ability of adult salmon may be affected. Additional behavioural studies on salmon homing are required to investigate how our observed reduction in olfactory responses is translated into behaviour. Furthermore, exposure to weak freshwater acidification (pH 6.1–6.3) has been shown to suppress spawning and migration behaviours in sockeye salmon, *O. nerka*<sup>38–40</sup>, thereby impacting various life history traits<sup>41</sup>. However, previous freshwater studies on anti-predator behaviour and olfactory responses were conducted at significantly lower pH than our present study and without the use of  $\text{CO}_2$  (based on our water composition, a pH of 6.0–6.3 would correspond to a  $p_{\text{CO}_2}$  of  $\sim 4,000-9,000 \mu\text{atm}$ ), which may not be relevant in the context of climate change. To tease apart pH versus  $\text{CO}_2$  effects, research investigating both  $p_{\text{CO}_2}$  and pH effects in varying types of freshwater sources is required to better understand the mechanistic basis for impaired olfaction.

Additional behavioural alterations in pink salmon, such as decreased anxiety and increased boldness, may further compound the negative effects of elevated  $\text{CO}_2$  on predator avoidance behaviour, and thus survival. In our study, pink salmon fry reared in high  $\text{CO}_2$  in freshwater spent significantly more time in the



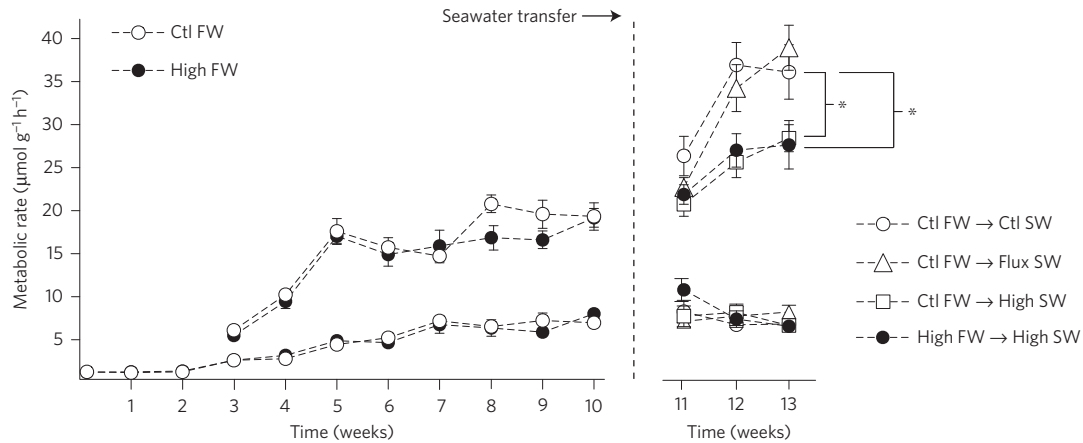
**Figure 5 | Absolute growth rates in pink salmon in the first two weeks following seawater transfer at different  $p_{\text{CO}_2}$  tensions.** High FW  $\rightarrow$  High SW represent fish reared in high  $\text{CO}_2$  in freshwater (2,000  $\mu\text{atm}$ ) and transferred into high  $\text{CO}_2$  in seawater (1,600  $\mu\text{atm}$ ). The remaining three groups were all reared in control freshwater (450  $\mu\text{atm}$ ) and transferred into one of three different seawater treatments of 450, 1,600 and a diurnal 450–1,600  $\mu\text{atm}$   $\text{CO}_2$  treatment (Ctl FW  $\rightarrow$  Ctl SW; Ctl FW  $\rightarrow$  High SW; Ctl FW  $\rightarrow$  Flux SW; respectively). Values are means  $\pm$  s.e.m. ( $n=8$ ). Asterisks indicate a statistically significant difference from the 450  $\mu\text{atm}$  control group ( $p < 0.05$ ).

centre zone around the novel object and less time in the thigmotaxis zone than control fish, suggesting that elevated  $\text{CO}_2$  may suppress anxiety. Thigmotaxis is a common index of anxiety-like behaviour in zebrafish and in rodents<sup>21,42</sup>. Inhibition of the GABAergic system with anxiogenic drugs leads to increases in anxiety-like behaviour<sup>5,42–44</sup>. Similarly, our results indicate significant increases in anxiety in both controls and high- $\text{CO}_2$ -reared fish after treatment with gabazine, with complete reversal of the anxiety-reducing effects of elevated  $\text{CO}_2$ . In ref. 4 it was also shown that gabazine reversed the effects of  $\text{CO}_2$  on olfactory ability and behavioural lateralization in larval damselfish and clownfish, and a mechanistic explanation was proposed for the effects of OA; a reversal in chloride and bicarbonate gradients through  $\text{GABA}_A$  receptors, which shifts some of these receptors from inhibitory to excitatory. Consistent with this altered  $\text{GABA}_A$  receptor activity, exposure to high  $\text{CO}_2$  increased anxiety in juvenile rockfish (*Sebastes diploproa*) and treatment with gabazine resulted in increased anxiety in the control fish to levels of high- $\text{CO}_2$ -exposed fish<sup>5</sup>. In marine stickleback, exposure to elevated  $\text{CO}_2$  reduced boldness and curiosity during a novel approach test<sup>6</sup>. Unlike stickleback, pink salmon exposed to high  $\text{CO}_2$  spent more time around the novel object, demonstrating an increase in boldness. The observed  $\text{CO}_2$ -induced differences in anxiety and boldness in seawater compared to the freshwater salmon in this study may be due to a variety of reasons, from completely different mechanisms that regulate ion balance to differences in life history traits.

### Growth and metabolism in seawater

Our results show that future increases in  $\text{CO}_2$  in seawater may impair early seawater survival of migrating pink salmon fry through impacts on subsequent growth and MMR. Growth rates were negative in groups transferred into high- $\text{CO}_2$  seawater, whereas control fish continued to grow throughout our seawater exposure. Fish were not fed; however, internal yolk stores were still present,





**Figure 6 | RMR and MMR during development in pink salmon in freshwater and following seawater transfer at different  $p_{\text{CO}_2}$  tensions.** RMR (lower lines) and MMR (upper lines) of 450  $\mu\text{atm}$ -reared fish (Ctl FW) and 2,000  $\mu\text{atm}$ -reared fish (High FW) during development in freshwater (left) and post-seawater transfer (right). See Figure 5 title for further details on seawater transfers. Values are means  $\pm$  s.e.m. ( $n=6-8$ ). Asterisks indicate statistically significant differences between indicated groups throughout the entire seawater exposure ( $p < 0.05$ ).

which were presumably drawn on for growth. This suggests that tissue production may be impaired at high  $\text{CO}_2$ . However, because growth rates are based on total wet mass, the inability to hypo-osmoregulate efficiently at high  $p_{\text{CO}_2}$  could also have contributed to the apparent negative growth rate estimates (due to water loss) during the transition to seawater. If the lower growth rates at high  $p_{\text{CO}_2}$  are associated with impaired hypo-osmoregulatory ability, then the osmoregulatory challenge of seawater entry may be further compounded by OA. Following seawater transfer, an increase in  $\text{CO}_2$  reduced MMR in pink salmon fry. These results indicate that pink salmon fry may be particularly sensitive to climate-change-related acidification during exercise, which may have implications on the success of their seaward migration and early ocean survival. Pink salmon migrate to sea soon after emergence and this heightened capacity for activity may be crucial to their survival in a predator-dense environment. However, elevated  $p_{\text{CO}_2}$  may reduce the capacity for maximal  $\text{O}_2$  uptake and exercise of migrating fry, making them more susceptible to predation and reducing their foraging success at a time when yolk reserves become limiting.

### Present and future implications for pink salmon

This study demonstrates that pink salmon may be faced with numerous sublethal impacts of acidification on their physiology and behaviour under predicted future increases in  $p_{\text{CO}_2}$ . However, carbonate chemistry data of inland waters suggests that some freshwater ecosystems are at present exposed to temporal elevations in  $p_{\text{CO}_2}$  (ref. 45). For example,  $p_{\text{CO}_2}$  in the Columbia River, which hosts runs of various salmon species, ranges from 541 to 981  $\mu\text{atm}$  (ref. 46). Similarly, recent oceanic surveys of the West Coast of North America suggest that our coastal ecosystems temporally experience large elevations in  $\text{CO}_2$ , which may exceed 1,000  $\mu\text{atm}$  and persist for months in some locations<sup>20,47-49</sup>. In Puget Sound, a large West Coast estuary,  $p_{\text{CO}_2}$  values exceed mean atmospheric levels and can occasionally reach 2,500  $\mu\text{atm}$  during autumn near-surface waters<sup>20</sup>. Therefore, near-shore marine environments, which provide refuge to schools of pink salmon before they double in size and begin their offshore migration<sup>14</sup>, may also be subjected to large variations in  $p_{\text{CO}_2}$ . Long-term records of pH in a localized region of the Strait of Georgia show pH regularly dropping to 7.5 from the year 2001 and onwards<sup>50</sup>, which corresponds to a  $p_{\text{CO}_2}$  estimate of 1,600  $\mu\text{atm}$ , at which level our study found detriments to growth and aerobic performance in pink salmon. It is important to note that  $p_{\text{CO}_2}$  in freshwater and coastal marine systems may vary greatly seasonally and spatially<sup>18,20,46</sup>. Nevertheless, our results indicate significant dose-dependent negative effects of  $\text{CO}_2$  that

span the range of present and future projected increases in  $\text{CO}_2$  at a very critical time in their life history, freshwater development and early ocean entry. Given this species' central ecosystem role in freshwater, marine and terrestrial food webs and their important economic and culture roles in Aboriginal communities, continued increases in  $\text{CO}_2$  may have widespread implications on ecosystem productivity and the many communities they support. Clearly, more research is required to investigate the potential impact on other salmonid species in particular, and other freshwater fish in general, which represent almost half of the world's fish species. This will permit a more comprehensive understanding of the potential impact of climate-change-related acidification on freshwater ecosystems, for which little is known, despite their great biodiversity.

### Methods

Methods and any associated references are available in the [online version of the paper](#).

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## Author contributions

M.O. and C.J.B. devised the study. M.O., C.J.B., J.E., T.J.H. and S.-S.Y. designed the experiments. M.O., T.J.H., J.E., E.M.L., J.G., A.J. and J.L. conducted the experiments. M.O., E.M.L., J.E. and T.J.H. developed equipment. M.O. and E.M.L. collected water samples and conducted water analyses. M.O. and C.J.B. wrote the manuscript. M.O., C.J.B., T.J.H., J.E., S.-S.Y. and D.A.C. contributed to intellectual input and edited this manuscript. All authors approved this manuscript.

## Additional information

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## Competing financial interests

The authors declare no competing financial interests.

## Methods

**Freshwater development and seawater transfer.** Pink salmon-eyed embryos were transported from Quinsam River Hatchery (Campbell River, Vancouver Island) to the University of British Columbia. Embryos were incubated in total darkness at  $4.0 \pm 1.0^\circ\text{C}$  in small containers filled with dechlorinated Vancouver City tap water ( $[\text{Na}^+]$ , 0.17 mM;  $[\text{Cl}^-]$ , 0.21 mM; hardness,  $30 \text{ mg l}^{-1} \text{ CaCO}_3$ ). At 470 accumulated thermal units (ATU; days  $\times$  temperature in  $^\circ\text{C}$ ), embryos were pooled, sorted based on size, and then uniform-sized embryos (weight of  $169 \pm 14 \text{ mg}$  and diameter of  $7.0 \pm 0.7 \text{ mm}$ ) were transferred into their respective  $\text{CO}_2$  treatments in freshwater (present day control: 450;  $477 \pm 22 \mu\text{atm}$ ; 1,000:  $1,036 \pm 28 \mu\text{atm}$ ; 2,000:  $2,031 \pm 59 \mu\text{atm}$ ; and 24 h oscillating: 450–2,000  $\mu\text{atm}$ ). The 1,000  $\mu\text{atm}$  freshwater treatment was chosen to mimic end-of-century projections for atmospheric  $\text{CO}_2$  and present day  $\text{CO}_2$  levels in freshwater systems supersaturated with  $\text{CO}_2$  (ref. 45). The 2,000  $\mu\text{atm}$  freshwater treatment was chosen to mimic future elevated  $\text{CO}_2$  in freshwater systems supersaturated with  $\text{CO}_2$ . At 10 weeks post-hatch, fish nearing yolk sac absorption (YSA) and reared under control conditions were transferred into seawater at one of three  $p_{\text{CO}_2}$  tensions: control  $\text{CO}_2$  (450;  $448 \pm 11 \mu\text{atm}$ ), constant high  $\text{CO}_2$  (1,600;  $1,593 \pm 30 \mu\text{atm}$ ) or fluctuating  $\text{CO}_2$  (450–1,600  $\mu\text{atm}$ ). The 1,600  $\mu\text{atm}$   $\text{CO}_2$  level was chosen to mimic fish migrating into end-of-century projected  $\text{CO}_2$  coastal conditions or present day high local coastal  $\text{CO}_2$  conditions. Fish reared in high  $\text{CO}_2$  in freshwater were also transferred into high  $\text{CO}_2$  in seawater (1,600;  $1,593 \pm 30 \mu\text{atm}$ ) to mimic future elevated  $\text{CO}_2$  conditions in both environments (Supplementary Fig. 1).

**Growth and yolk consumption.** Measurements of developing pink salmon wet mass (wtm) and length ( $n=8$  for wet mass;  $n=8$  for length) were taken weekly from two weeks pre-hatch up until two weeks post-seawater transfer (11 weeks post-hatch). Three embryos or fish were sampled from two of the three replicates and two embryos or fish were sampled from the last replicate to give a sample size of eight. Embryos or fish were then fixed in 5% neutral buffered formalin for up to 21 days before the yolk was dissected from the fish to obtain yolk wtm and tissue wtm measurements. These samples were then dried in an oven at  $58^\circ\text{C}$  for at least five days to obtain dry weights for alevin tissue and yolk. Gross production efficiencies at week 10 were calculated as the increase in tissue dry mass divided by the loss in yolk dry mass multiplied by 100. Growth rates in seawater were calculated as the difference in alevin wtm at day 14 and at day 0 post-seawater transfer.

**Respirometry.** RMR and MMR were measured throughout development in freshwater and following seawater transfer. To accommodate the growing size of developing fish, three different-sized closed respirometers were used:  $3.41 \pm 0.04 \text{ ml}$  for week 1–4;  $7.43 \pm 0.10 \text{ ml}$  for week 5–10; and  $10.61 \pm 0.12 \text{ ml}$  for day 1–14 post-seawater transfer. Chambers were rinsed in Virkon Aquatic (Syndel Laboratories) at the end of each day to reduce background bacterial respiration, which was determined to be negligible from blank experiments. To ensure adequate water mixing, a mini stir bar was placed beneath a false bottom mesh in all the chambers. A total of eight fish per treatment (four treatments, where eight fish were randomly pooled from replicate tanks) per time point (weekly) were used for measurement of RMR and MMR in the water from the respective  $p_{\text{CO}_2}$  treatment in which the fish were reared.

RMR was measured weekly throughout development in freshwater and up until 14 days post-seawater transfer. Fish were placed in the chambers, where they were acclimated in darkness at an appropriate acclimation time (2 h for eggs, 3 h for alevin, 4 h for fry) in flow-through water of their respective  $p_{\text{CO}_2}$  treatments. Acclimation times were determined by preliminary testing, which indicated that longer periods did not significantly reduce RMR. After the acclimation period, the chamber was sealed and the decline in oxygen was monitored over time, using a fibre optic oxygen sensor (PreSens, Model NFSL) to calculate mass-specific RMR. Once  $\text{O}_2$  levels reached 60% saturation, the measurement was terminated and the chamber was re-supplied with flow-through water. The sensor was connected to a four-channel oxygen meter (PreSens, OYY-4 Micro), allowing the simultaneous recording of four chambers. Data was obtained using the accompanying software (OXY-4v2\_11TX), with temperature compensation. We referred to our resting metabolic rate measurements as routine and not standard metabolic rate to account for spontaneous movements from the fish in the chamber.

The maximum rate of oxygen uptake (MMR) immediately following an exhaustive chase protocol was used as an index of maximal  $\text{O}_2$  uptake capacity. MMR was measured weekly from week 3–10 in freshwater and from day 1–14 post-seawater transfer, using a chase protocol similar to the one outlined in ref. 51. Briefly following the determination of RMR, the fish was removed from the chamber, placed in a container filled with the respective  $p_{\text{CO}_2}$  water and then chased to exhaustion with a plastic pipette for a maximum of 4 min. The inability to right itself within 3 s following inversion was defined as complete exhaustion. At this point the fish was immediately returned to the chamber and MMR was measured (all within 30 s). MMR was calculated from the initial decrease in oxygen (within 5 min) following closure of the chamber. Although maximum sustained swimming speed ( $U_{\text{crit}}$ ) is typically used to obtain MMR in fish, previous studies have shown

that chase protocols can yield similar or higher values of MMR in some species of fish<sup>52–54</sup>. Burst swimming and exhaustive chase protocols are often used to estimate MMR in larval fish and species that are poor swimmers<sup>54–57</sup>. During the alevin stage (yolk present), pink salmon swim poorly and exhibit burst swimming, making  $U_{\text{crit}}$  (sustained swimming) tests a poor indicator of MMR. In larger and more developed pink salmon fry ( $\sim 2.81 \text{ g}$  versus the 0.16–0.20 g fish in our study),  $U_{\text{max}}$  (constant acceleration) and  $U_{\text{crit}}$  swim tests yielded similar fatigue speeds regardless of the type of test used, suggesting that similar metabolic processes were occurring during both protocols<sup>58</sup>. Similar results have been observed in swimming small fish, including other species of salmonids<sup>59,60</sup>. Early in development, fish may rely more on anaerobic metabolism during exercise while aerobic muscles are still developing<sup>60</sup>; thus, an indicator of maximal oxygen uptake capacity, as conducted here, will inform on both aerobic capacity and the rate of recovery following exhaustion.

**Alarm cue extraction for electro-olfactograms and two-channel choice test.** Skin and muscle tissue from pink salmon fry were used as a source of the alarm cue. Approximately 500 pink salmon fry were euthanized with tricaine methanesulphonate (MS-222, Western Chemicals) ( $0.5 \text{ g l}^{-1}$  of distilled water buffered with  $\text{NaHCO}_3$  to pH 7) and rinsed with distilled water before skin and muscle fillets were dissected from the fish. The skin and muscle fillets were homogenized, diluted with distilled water and filtered through filter floss to remove any large tissue particles. The skin extract was then diluted with distilled water to yield a stock solution with a concentration of 0.1 g of tissue per ml of water. The stock solution was frozen in 20 ml aliquots and stored at  $-20^\circ\text{C}$  until needed.

**Two-channel choice test.** A two-channel choice flume<sup>61</sup> was used to assess behavioural responses of pink salmon fry (week 11 of  $\text{CO}_2$  exposure) to conspecific alarm cues in freshwater. The flume allowed pink salmon to freely choose between two different sides of the flume that contained water from two different sources. One water source consisted of freshwater at the same  $p_{\text{CO}_2}$  tension that the fish was reared in, with alarm cue skin extract added to yield a final testing concentration of  $0.5 \text{ mg ml}^{-1}$ . The other source consisted of freshwater at the same  $p_{\text{CO}_2}$  tension, with distilled water added in place of the skin extract. Similar to the protocols in refs<sup>1,61</sup>, water from two different sources was gravity fed into the flume at a rate of  $100 \text{ ml min}^{-1}$ , which was set and maintained by flow controllers. Coloured dye was used to check for laminar flow at the end of trials. During each trial, a single fry was placed at the downstream end of the flume and allowed to acclimate for 2 min. Fry that did not actively swim during this acclimation period were discarded. After acclimation, the location of the head of the fry was recorded at the end of every 5 s interval for 2 min. The steps (including acclimation) were repeated after switching the sides from which the respective water sources were introduced into the choice flume to control for potential side preference. Fry were discarded after each trial.

**Alarm cue electro-olfactogram recording.** EOGs were conducted in freshwater on pink salmon fry at week 11 of the  $\text{CO}_2$  exposure ( $n=3$ ). Before EOG recording, the fish was anaesthetized in  $0.3 \text{ mg l}^{-1}$  tricaine methanesulphonate (MS-222, Western Chemicals) buffered with sodium bicarbonate for  $\sim 5 \text{ s}$ . Immediately afterwards, fish were placed in the EOG set-up and were constantly perfused with freshwater at the respective  $p_{\text{CO}_2}$  tension it was reared in for the duration of the experiment. Gelatin-filled glass capillary pipettes (8% agar in 0.8% NaCl) were placed to bridge the sensory organ of the animal to silver chloride (Ag/AgCl) microelectrodes filled with 3 M potassium chloride (KCl). Nasal bridge skin was gently removed with fine forceps to expose the olfactory epithelium. The recording and the reference electrodes (World Precision Instruments) were then positioned over the surface of the olfactory epithelium and the skin around the nasal cavity, respectively (Supplementary Fig. 2). The olfactory epithelium was constantly perfused with freshwater at the respective rearing  $p_{\text{CO}_2}$  tension ( $12.9 \text{ ml min}^{-1}$ ) while a timer-controlled solenoid valve (Model 655, GRALab) delivered test odors ( $430 \mu\text{l}/2 \text{ s}$ ) by switching between freshwater and the alarm cue dilutions. Amplified signals (ML132, ADInstruments) were visualized on the computer monitor through digitizing (ML866, ADInstruments) and filtering devices (low-pass, cutoff frequency 50 Hz). Freshwater at the respective  $p_{\text{CO}_2}$  tension was used as a negative control in EOG recording (Supplementary Fig. 2). Serial dilutions of the alarm cue skin extract were prepared by diluting the stock solution (0.1 g of tissue per ml of water) with freshwater at the respective rearing  $p_{\text{CO}_2}$ .

**Amino acid electro-olfactogram recording.** EOGs were conducted in freshwater on pink salmon alevin (yolk still present) at week 8 of the  $\text{CO}_2$  exposure ( $n=5$ ). Fish were anaesthetized and prepared for EOG recordings with the same procedures and equipment as described above (Supplementary Fig. 2). After positioning the recording electrodes, each fish was perfused with freshwater at the respective rearing  $p_{\text{CO}_2}$  tension (control or 2,000  $\mu\text{atm}$ ) for 30 min. After this period of acclimation, electrophysiological responses to nine different amino acids at the olfactory epithelium were recorded (Supplementary Fig. 3). The nine amino acids selected (L-arginine, L-asparagine, L-aspartic, L-cysteine, L-glutamic, L-glutamine, L-histidine, L-lysine, L-threonine) showed relatively large response magnitudes



compared to all other amino acids, and thus were selected for EOG testing in pink salmon fry. The olfactory epithelium was constantly perfused with freshwater at the respective rearing  $p_{\text{CO}_2}$  tension ( $12.9 \text{ ml min}^{-1}$ ) while a timer-controlled solenoid valve (Model 655, GRALab) delivered test odorants ( $430 \mu\text{l}/2 \text{ s}$ ) by switching between the respective freshwater type and a particular amino acid dilution. After recordings in rearing conditions, control fish were transferred into  $2,000 \mu\text{atm}$  freshwater and  $2,000 \mu\text{atm}$ -reared fish were transferred into  $450 \mu\text{atm}$  freshwater for 2 h. After 2 h of acclimation, EOG responses to the same nine amino acids were recorded on control and  $2,000 \mu\text{atm}$ -reared fish.

Stock solutions of amino acids (MAK002-1KT, Sigma-Aldrich) were prepared in deionized water and stored at  $4^\circ\text{C}$ . Freshwater at the  $p_{\text{CO}_2}$  tension that was being tested was used to dilute the amino acid odorants ( $10^{-3} \text{ M}$ ) for all EOG experiments.

**Novel approach test.** The novel approach test consisted of an arena, 31 cm in diameter, and a small Lego figurine, which was placed in the centre of the arena<sup>5</sup>. Before each trial, the arena was filled to a height of 10 cm with new freshwater at the respective rearing  $p_{\text{CO}_2}$  tension ( $450$ ,  $1,000$  or  $2,000 \mu\text{atm}$ ). The arena was divided into three virtual zones (centre, middle and thigmotaxis zone; Supplementary Fig. 4) and time spent in each zone was measured using ETHOVISION XT (v. 9.0, Noldus) motion tracking software. A single fish was placed in the arena and released from the net into the middle zone, facing the object, and allowed to freely swim out of the net. Trials were initiated approximately 5 s after the fish was placed in the arena. For the gabazine treatments, only fish reared at  $450$  or  $2,000 \mu\text{atm}$  were tested. Fish were individually placed in a solution of gabazine ( $4 \text{ mg l}^{-1}$ ,  $10.9 \mu\text{M}$ ) dissolved in the respective freshwater  $p_{\text{CO}_2}$  for 30 min. Following this, fish were introduced to the arena for testing at the respective  $p_{\text{CO}_2}$ . Each fish was tested only once.

**Statistical analysis.** Data are expressed as means  $\pm$  s.e.m., and were log-transformed when necessary to meet the assumptions of normality and equal variance. For all tests, a significance level of  $p < 0.05$  and a confidence interval of 95% was used.

One-way nested ANOVAs were used to analyse wet mass, dry mass, length, production efficiency, growth rates and behavioural responses to alarm cue data using the software SAS JMP 11 (Version 11.2, SAS Institute). Subjects were nested within their replicate tank, which was nested within their respective treatment. Pairwise comparisons among treatments and comparisons to the control were made using the TukeyHSD and Dunnett *post hoc* test when significance was found.

Mixed effects models with time as a repeated factor were used to analyse log-transformed mass-specific RMR and mass-specific MMR data using the program SAS JMP Pro 11 (Version 11.2, SAS Institute). Time and treatment were treated as fixed effects and tank (nested within treatment) was treated as a random effect. As there were no significant interaction effects (time  $\times$  treatment) on mass-specific RMR and mass-specific MMR, the interaction term was removed from our mixed models. Preliminary two-way ANOVAs including log-transformed mass as a factor showed no significant effects of mass on mass-specific RMR and MMR; as a result, mass was dropped from our models. This is consistent with the finding that, early in development, RMR and MMR scale isometrically with body mass<sup>44,46</sup>. All RMR and MMR data are expressed as mass-specific metabolic rates. Comparisons to the control group using the Dunnett *post hoc* test were made when significance was detected.

A repeated measures ANOVA was used to analyse alarm cue EOG dose–response–curve data with SPSS Statistics 21 (Version 21.0; IBM). Greenhouse–Geisser adjusted  $F$  values were used because the assumptions of sphericity were not met. Comparisons among groups were made using the Sidak method for *post hoc* testing. Student's paired  $t$ -tests were used to compare EOG responses to each amino acid in freshwater for control fish before and after transfer to  $2,000 \mu\text{atm}$  and  $2,000 \mu\text{atm}$ -reared fish before and after transfer to  $450 \mu\text{atm}$   $\text{CO}_2$ . Comparisons of EOG responses before transfers were also made between control and  $2,000 \mu\text{atm}$ -reared fish using Student's  $t$ -tests. All amino acid EOG data were analysed with the program SigmaPlot (Version 12.0; SYSTAT Software).

Data from our novel approach test were not normally distributed; thus, non-parametric tests were performed (SAS JMP 11). Times spent in different zones were analysed using the Dunn method (with control) for multiple comparisons or using the Wilcoxon test for gabazine-treated comparisons (+gabazine).

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