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SHORT COMMUNICATION

Water pH limits extracellular but not intracellular pH compensation in the CO_2-tolerant freshwater fish *Pangasianodon hypophthalmus*

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ABSTRACT

Preferentially regulating intracellular pH (pH_i) confers exceptional CO_2 tolerance on fish, but is often associated with reductions in extracellular pH (pH_e) compensation. It is unknown whether these reductions are due to intrinsically lower capacities for pH_e compensation, hypercarbia-induced reductions in water pH or other factors. To test how water pH affects capacities and strategies for pH compensation, we exposed the CO_2-tolerant fish *Pangasianodon hypophthalmus* to 3 kPa P_{CO_2} for 20 h at an ecologically relevant water pH of 4.5 or 5.8. Brain, heart and liver pH_i was preferentially regulated in both treatments. However, blood pH_e compensation was severely reduced at water pH 4.5 but not 5.8. This suggests that low water pH limits acute pH_e but not pH_i compensation in fishes preferentially regulating pH_i. Hypercarbia-induced reductions in water pH might therefore underlie the unexplained reductions to pH_e compensation in fishes preferentially regulating pH_i, and may increase selection for preferential pH_i regulation.

KEY WORDS: Acid–base, Hypercarbia, *Pangasius*, Bicarbonate

INTRODUCTION

The aquatic partial pressure of carbon dioxide (P_{CO_2}) in tropical river basins can be driven above 6 kPa daily by microbial respiration and organic decay (Furch and Junk, 1997). These rapid elevations in P_{CO_2} exceed atmospheric levels by over 200-fold, and impose a severe acute respiratory acidosis on fish as CO_2 diffuses from the water into their blood and tissue (Heisler, 1984). Despite the extreme nature of these rapid acidoses, many fishes routinely endure this challenge, as evidenced by the high levels of species richness and abundance in these environments (Dudgeon et al., 2006).

Coupled pH regulation ($pH_{coupled}$) and preferential intracellular pH regulation (pH_{pi}) are two strategies fish use to compensate for an acute respiratory acidosis (Shartau et al., 2016). These strategies represent endpoints of a continuum along which rates and degrees of intracellular pH (pH_i) and extracellular pH (pH_e) compensation vary.

In $pH_{coupled}$, tissue pH_i is coupled to blood pH_e. During an acidosis event, pH_i and pH_e both fall and recover together along similar trajectories within 24–48 h. Coupled recovery of pH_i and pH_e requires transepithelial exchange of acid–base relevant ions for net acid excretion and/or base uptake (Stewart, 1978; Claiborne et al., 2002). The exchange of chloride for bicarbonate and/or sodium for protons is believed to primarily drive this recovery, but full compensation is generally associated with an increase in plasma bicarbonate balanced by an equimolar reduction in plasma chloride (Heisler, 1984; Brauner and Baker, 2009).

In pH_{pi}, pH_i is not coupled to pH_e. Within minutes of CO_2 exposure, pH_i is at or above control levels despite large reductions in pH_e (Baker, 2010). Additionally, pH_e recovery is often incomplete or absent within 24–48 h (Brauner et al., 2004). Here, pH_i is maintained by the exchange of acid–base relevant ions between intracellular and extracellular compartments whether pH_e compensation occurs or not (Brauner and Baker, 2009; Occhipinti and Boron, 2015), and reductions in the rate and degree of acute pH_e compensation remain unexplained.

Why fishes express $pH_{coupled}$ or pH_{pi} is unclear. However, severe acute hypercarbia is hypothesized to select for pH_{pi} by exceeding the capacity and/or limiting the rate of acute pH_e compensation required for $pH_{coupled}$ to defend pH_i (Shartau et al., 2016). Indeed, full pH_e compensation within 24–48 h of hypercarbia is limited to ∼2 kPa P_{CO_2} in most freshwater fishes tested, while many fishes expressing pH_{pi} can robustly defend pH_i above 6 kPa P_{CO_2} without pH_e compensation (Brauner and Baker, 2009; Shartau et al., 2016). One hypothesis for this apparent limit to acute pH_e compensation suggests many fishes are unable to elevate plasma bicarbonate above the ∼25–30 mmol l^{-1} required for full pH_e recovery at ∼2 kPa P_{CO_2}, let alone the ∼100–150 mmol l^{-1} required at ∼6 kPa (Heisler, 1984; Brauner and Baker, 2009). A second hypothesis posits that water ion composition reduces the rate and/or degree of pH_e compensation by creating unfavourable trans-epithelial gradients for acid–base relevant ion exchange (Larsen and Jensen, 1997). Indeed, most CO_2 exposures exceeding the capacity for acute pH_e compensation in freshwater fishes also reduce water pH below 5.3, which is proposed to thermodynamically inhibit net proton excretion in rainbow trout at ambient P_{CO_2} (Lin and Randall, 1995). Despite supporting evidence for both hypotheses, neither has been directly tested for a role in limiting pH_e compensation and selecting for pH_{pi} during acute hypercarbia.

The Mekong catfish *Pangasianodon hypophthalmus* was recently reported to fully compensate pH_e at 4 kPa P_{CO_2} (Damsgaard et al., 2015). Compensation was associated with a surprising ∼45 mmol l^{-1} increase in plasma bicarbonate within 48 h of exposure. This elevated capacity for acute pH_e compensation suggests that *P. hypophthalmus* might express $pH_{coupled}$ rather than pH_{pi} to defend pH_i in acute hypercarbia above 2 kPa P_{CO_2}. This would be in stark contrast to findings for 19 of 20 CO_2-tolerant freshwater fishes tested (Shartau et al., 2016), including the Amazonian

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catfish *Pterygoplichthys pardalis*, which expresses pH_{pi} and negligible pH_e compensation at 1–6 kPa P_{CO_2} (Brauner et al., 2004). However, pH_i in *P. hypophthalmus* was not examined for preferential regulation, and water pH during hypercarbic exposure was 5.8 (Damsgaard et al., 2015). This is well above the proposed threshold water pH of 5.3 for net proton excretion in rainbow trout, and much higher than water pH in the *P. pardalis* study (water pH 4.5 at 4 kPa P_{CO_2}; Brauner et al., 2004).

We therefore sought to answer two questions. First, is the exceptional capacity for acute pH_e compensation in *P. hypophthalmus* limited by a lower, more common hypercarbic water pH? Second, if pH_e compensation is limited by low water pH, can *P. hypophthalmus* express pH_{pi} like most other CO_2-tolerant freshwater fishes tested? To address these questions, we measured pH_e and pH_i in *P. hypophthalmus* during exposure to 3 kPa P_{CO_2} for 20 h in water artificially held at pH 4.5 or 5.8. Our results provide further insight into the factors limiting $pH_{coupled}$ and selecting for pH_{pi}.

MATERIALS AND METHODS

Animal husbandry

Pangasianodon hypophthalmus (Sauvage 1878) were obtained from a local fish supplier in Can Tho, Vietnam and kept at Can Tho University for 3 months prior to experimentation. Fish were held in aerated 3000 l tanks fitted with a recirculating biofiltration system and kept on a 12 h:12 h light:dark photoperiod. Water Cl^- and pH in these holding conditions were 0.35 mmol l^{-1} and 7.2±0.1, respectively, which is similar to that listed for native habitat in the nearby Mekong River (in mmol l^{-1}: $[Cl^-]$ 0.28, $[Na^+]$ 0.39, $[Ca^{2+}]$ 0.63, $[Mg^{2+}]$ 0.33, $[CaCO_3]$ 0.53, pH 7.2; Ozaki et al., 2014; Kongmeng and Larsen, 2014). Fish were fed to satiation once daily with commercial dry pellets obtained from a local supplier and held under these conditions for at least 3 weeks prior to experimentation. Fish wet mass ranged between 50 and 100 g. All husbandry and experimentation were performed in accordance with national guidelines for the protection of animal welfare in Vietnam as well as the University of British Columbia Animal Use Protocol (AUP) no. A11-0235.

Protocol and measurements

One day prior to experimentation, fish were randomly transferred from holding tanks to a 200 l aerated experimental tank kept at 28°C. On the day of experimentation, fish were exposed to 3 kPa P_{CO_2} in water at a pH of either 5.8 or 4.5 for up to 20 h. Water pH of 5.8 was achieved by bubbling 3% CO_2 into the aerated experimental water at trial onset. Water pH of 4.5 was achieved by simultaneously introducing sulfuric acid (H_2SO_4) into the aerated experimental water while bubbling with 3% CO_2. pH 4.5 was chosen as the lower water pH because it matches that of a previous study where the Amazonian catfish *P. pardalis* was exposed to 3 kPa P_{CO_2} (Brauner et al., 2004). The desired P_{CO_2} and water pH for each treatment were reached within 15 min of trial onset. Sulfuric acid was used to avoid introducing ions, such as Na^+ and Cl^-, which may confound the effects of water pH on acid–base regulation. Water P_{CO_2} and pH were monitored continuously using an Oxyguard Pacific system fitted with a G10ps CO_2 probe and a K01svpld pH probe (Oxyguard International A/S, Farum, Denmark). The G10ps probe measures P_{CO_2} independently of water pH, such that measurements are not confounded by pH changes in the experimental treatments. A mix of CO_2 and air was regulated by the Oxyguard system to reach and maintain a constant water P_{CO_2} of 3 kPa (±0.02 kPa) and full oxygen saturation. Fish were terminally sampled (see below) following 0, 3 and 20 h exposure to 3 kPa P_{CO_2} in both water pH treatments.

Prior to sampling, fish were rapidly transferred (<1–2 s) from experimental tanks by net to a neighbouring 20 l tank containing a lethal concentration of benzocaine (100 mg l^{-1} benzocaine in 3 ml of 70% ethanol), which was darkened and covered to reduce struggling. Following cessation of gill ventilation (<2 min), a 0.5 ml blood sample was collected by caudal puncture with a heparinized syringe. Blood samples were subsequently divided into two aliquots, one of which was immediately measured for pH_e. The spinal cord was then severed, and tissues (heart, liver and brain) were excised, wrapped in pre-labelled aluminium foil and frozen in liquid nitrogen. This entire procedure was completed within 2 min of ventilatory arrest. The second blood aliquot was centrifuged for 3 min at 6000 rpm to separate plasma and red blood cells (RBCs). Plasma and RBCs were frozen in liquid nitrogen with the tissue samples, and all samples were subsequently transferred to −80°C for storage until further analysis.

pH_e, pH_i and water pH were measured with a Radiometer Analytical SAS pH electrode (GK2401C; Villeurbanne, France) connected to a Radiometer PHM84 pH meter (Copenhagen, Denmark) thermostatically set to 28°C to match the water temperature of the experiments. RBC pH_i was measured according to the freeze–thaw method (Zeidler and Kim, 1977), and tissue pH_i was measured according to the metabolic inhibitor tissue homogenate method (Portner et al., 1990; McKenzie et al., 2003; Baker et al., 2009b). Total CO_2 (TCO_2) was measured in plasma (Corning 965 CO_2 analyser, Essex, UK). Blood P_{CO_2} and plasma $[HCO_3^-]$ were calculated from pH_e and TCO_2 with the Henderson–Hasselbalch equation. CO_2 solubility (αCO_2) and pK′ values were taken from Boutilier et al. (1984).

Statistics

Data were analysed with Prism 5 for Mac OS X (Version 5.0a; GraphPad Software, Inc.). Means for each metric were compared within treatments and across time with one-way ANOVA and Tukey's *post hoc* test ($P<0.05$). All data are presented as means±s.e.m.

RESULTS AND DISCUSSION

After 3 h of hypercarbia, pH_e fell dramatically in both treatments, as expected. The increased blood P_{CO_2} reduced pH_e from 7.79±0.02 to 7.40±0.03 and 7.45±0.012 in pH 5.8 and pH 4.5 water, respectively ($P<0.01$; Fig. 1). Furthermore, pH_e in both treatments fell below the blood non-bicarbonate buffer line (Fig. 1). This suggests a metabolic component to the extracellular acidosis in both treatments, but plasma lactate concentration did not increase (Table 1). Thus, this metabolic component was instead probably due to a net exchange of HCO_3^- and/or H^+ between the intracellular and extracellular compartments, which is consistent with pH_{pi} expression (Heisler, 1982; Baker et al., 2009a).

After 20 h of hypercarbia, there was evidence for pH_e compensation in pH 5.8 water but little in pH 4.5 water. In pH 5.8 water, pH_e recovered by ∼40% from 3 h (Fig. 1, $P<0.05$) as plasma $[HCO_3^-]$ doubled to exceed the blood buffer line by ∼9 mmol l^{-1} at the respective P_{CO_2} (Fig. 1, $P<0.01$). In contrast, pH_e in pH 4.5 water did not recover significantly from 3 h (Fig. 1), and plasma $[HCO_3^-]$ did not exceed the blood buffer line (Fig. 1).

Tissue pH_i of brain, heart and liver was preferentially regulated in both pH 5.8 and pH 4.5 water (Fig. 2), but there was variation between tissues and treatments. Brain pH_i increased from control after 3 h of hypercarbia in both treatments ($P<0.05$) and remained elevated at 20 h (Fig. 2). In contrast, heart and liver pH_i did not differ significantly from controls in either treatment at any time. However, heart and liver pH_i did differ within their respective tissues between 3 and 20 h in the pH 5.8 water treatment (Fig. 2, $P<0.05$). Thus, brain pH_i appears more

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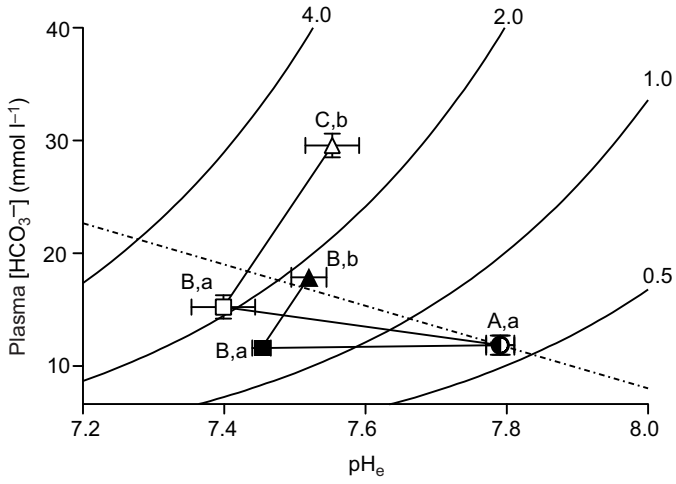

Fig. 1. Extracellular acid–base status in *Pangasianodon hypophthalmus* during exposure to 3 kPa P_{CO_2}. Extracellular blood pH (pH_e) versus plasma [HCO_3^-] after 0 h (circles), 3 h (squares) and 20 h (triangles) exposure to 3 kPa P_{CO_2} at water pH 4.5 (filled symbols) or pH 5.8 (open symbols). Dashed and curved lines represent the blood non-bicarbonate buffer line and P_{CO_2} isopleths in kPa, respectively. Data are presented as means±s.e.m. Uppercase and lowercase letters indicate significant differences within treatments for blood pH and plasma [HCO_3^-], respectively (n=8, one-way ANOVA, P<0.05).

robustly defended than that of heart and liver, and heart and liver pH_i appears more tightly regulated in pH 4.5 water than in pH 5.8 water. The latter difference could be attributed to a greater acidosis associated with higher *in vivo* P_{CO_2} in pH 5.8 water (Fig. 1), but this remains unknown. RBC pH_i fell with pH_e at 3 h in both treatments (Fig. 2), and did not recover within 20 h despite significantly increasing in pH 4.5 water. Lack of RBC pH_i regulation has been observed in all fishes expressing pH_{pi} to date (Shartau et al., 2016) and is consistent with the absence of β-adrenergically stimulated Na^+–H^+ exchange in Siluriformes (Berenbrink et al., 2005; Phuong et al., 2017). Despite this variation, the observed patterns in pH_i across all tissues in both treatments were typical of pH_{pi} expression (Shartau et al., 2016), and are corroborated by the reduction in plasma [HCO_3^-] below the blood buffer line observed after 3 h of hypercarbia in both treatments.

Our results show that the exceptional rate and degree of acute pH_e compensation in *P. hypophthalmus* is severely limited at a water pH of 4.5. Furthermore, *P. hypophthalmus* expresses pH_{pi} rather than $pH_{coupled}$ whether pH_e compensation occurs or not. As discussed below, this suggests hypercarbia-induced reductions in water pH may underlie previously unexplained reductions to the rate and degree of pH_e compensation in fishes expressing pH_{pi}. Variation in buffering capacity of the surrounding water might therefore mask higher, more similar rates and degrees of acute pH_e compensation across teleosts than previously believed, and low water buffering capacity may increase selection for pH_{pi} at P_{CO_2} normally within the limits of acute pH_e compensation and $pH_{coupled}$.

Table 1. Plasma lactate concentration of *Pangasianodon hypophthalmus* after 0, 3 and 20 h in 3 kPa P_{CO_2} at water pH 5.8 or 4.5

	Plasma lactate (mmol l^{-1})	
Time (h)	pH 5.8 water	pH 4.5 water
0	1.71±0.31	1.71±0.31
3	1.80±0.16	1.72±0.12
20	1.60±0.44	1.42±0.20

Data are presented as means±s.e.m. There were no significant differences from 0 h within treatments (n=8, one-way ANOVA, P>0.05).

Impaired pH_e compensation in *P. hypophthalmus* at a water pH of 4.5 is associated with an absence of net transepithelial exchange of acid–base relevant ions. Low water pH is hypothesized to inhibit bicarbonate uptake and proton excretion by creating unfavourable transepithelial gradients for ion transport machinery (Parks et al., 2010) and/or directly impairing transporter structure–function (Kwong et al., 2014). Indeed, inhibition of transepithelial ion flux by low water pH at ambient P_{CO_2} has been shown in several fishes (Freda and McDonald, 1988; Shartau et al., 2017; Ultsch, 1988). Although not tested here, similar thermodynamic and/or structure–function effects on ion transport could be limiting pH_e compensation in *P. hypophthalmus*. However, many fishes adapted to low pH environments still regulate plasma ions (Kwong et al., 2014). Thus, determining whether and how these fishes might compensate pH_e at low water pH also merits future study.

Surprisingly, this study is the first to directly test the isolated effects of water pH on acid–base regulation in fishes during acute hypercarbia. Previous studies have shown that acute pH_e compensation is also affected to a lesser degree by variation in water hardness and ion composition (Larsen and Jensen, 1997; Tovey and Brauner, 2018). However, logistical constraints precluded manipulating individual ions and controlling for pH in these studies. As a result, water pH differed by 1.5 units between treatments in some cases, and higher water pH was always associated with higher rates and degrees of pH_e compensation. In light of our findings, revisiting these experiments while controlling for water pH would be of interest, helping to further disentangle the effects of pH from those of other ions on acid–base regulation in fish.

Fish expressing pH_{pi} often exhibit reduced rates and degrees of acute pH_e compensation relative to those expressing $pH_{coupled}$ (Shartau et al., 2016). Furthermore, the approximate limit of 2 kPa P_{CO_2} for acute pH_e compensation observed in many freshwater teleosts expressing $pH_{coupled}$ (Heisler, 1984; Brauner and Baker, 2009) is much less than the 3–4 kPa limit observed in many marine teleosts (Hayashi et al., 2004; Perry et al., 2010). However, we show that low water pH during hypercarbia inhibits acute pH_e compensation in a freshwater fish expressing pH_{pi} to a rate and degree equal to that of marine teleosts. This suggests low water pH might underlie previously observed reductions in the rate and degree of acute pH_e compensation in other fishes expressing pH_{pi}. Further, it suggests that all teleosts, whether expressing pH_{pi} or $pH_{coupled}$ and whether freshwater or marine, might possess similarly high capacities for acute pH_e compensation. Indeed, differences in water buffering capacity could underlie much of the observed variation in these traits. Most fishes expressing pH_{pi} are investigated in the poorly buffered waters of their native tropical river basins (Shartau and Brauner, 2014), where modest hypercarbia dramatically reduces water pH (pH 4.5 at 3 kPa P_{CO_2}, Rio Blanco, Brazil; Gonzalez et al., 2005). These tropical waters are more poorly buffered than those in which fishes expressing $pH_{coupled}$ are typically tested (pH 5.5 at 3 kPa P_{CO_2} in Vancouver city water, Canada; Shartau et al., 2017), and both have a lower pH than seawater (pH 6.9 at 3 kPa P_{CO_2}; Hayashi et al., 2004). Other studies further support this hypothesis. For example, freshwater rainbow trout express $pH_{coupled}$ and typically have a limit of ∼2 kPa P_{CO_2} for acute pH_e compensation (Wood and LeMoigne, 1991; Brauner and Baker, 2009). However, rainbow trout exposed to hypercarbia in water at pH 6.9 fully compensated pH_e at ∼3 kPa P_{CO_2} within 24–48 h (Dimberg, 1988; Larsen and Jensen, 1997). This was accomplished by a net 45 mmol l^{-1} increase in plasma bicarbonate, matching that observed in *P. hypophthalmus* and marine teleosts. Thus, low water buffering capacity may mask shared, higher capacities for acute pH_e compensation closer to 3–4 kPa P_{CO_2} across teleosts.

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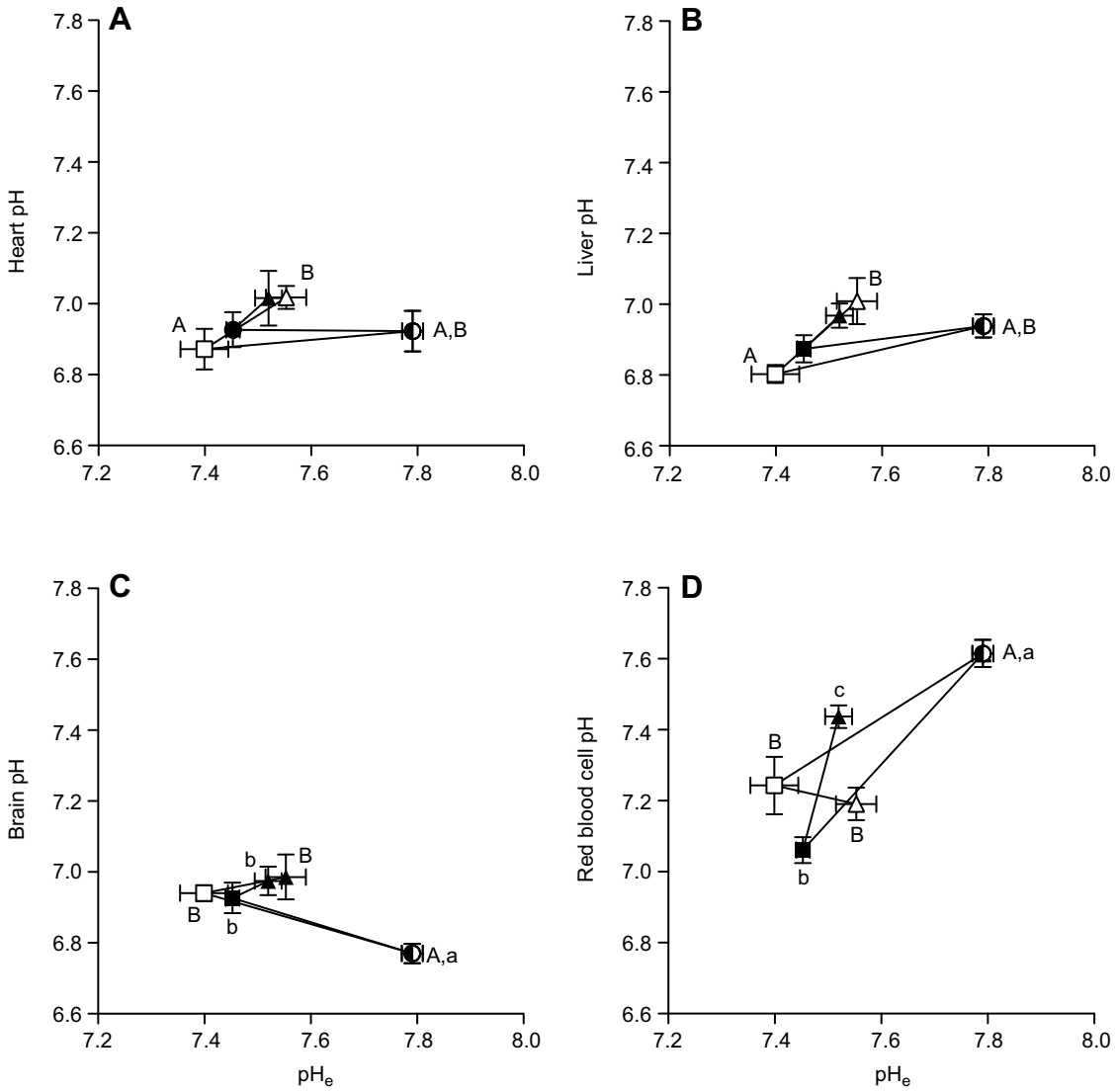

Fig. 2. Intracellular pH of *P. hypophthalmus* during exposure to 3 kPa P_{CO_2}. Extracellular blood pH (pH_e) versus intracellular pH (pH_i) of heart (A), liver (B), brain (C) and red blood cells (D) after 0 h (circles), 3 h (squares) and 20 h (triangles) exposure to 3 kPa P_{CO_2} at water pH 4.5 (filled symbols) or pH 5.8 (open symbols). Data are presented as means±s.e.m. Lowercase and uppercase letters indicate significant differences for pHi in pH 4.5 and pH 5.8 water, respectively (n=8, one-way ANOVA, P<0.05).

We are also the first to observe pH_{pi} expression in the presence and absence of acute pH_e compensation at the same P_{CO_2} in one species. This preference to regulate pH_e despite the ability to independently maintain pH_i suggests that even fishes expressing pH_{pi} may incur performance costs in the absence of pH_e compensation. The nature of these costs remains unknown, but if low water pH inhibits transepithelial ion transport as discussed, other vital processes relying on the same ion transport pathways could be impacted (e.g. osmoregulation, ammonia excretion, RBC function, etc.). This finding suggests that fishes expressing pH_{pi} in low water pH during hypercarbia might incur additional performance costs relative to those expressing pH_{pi} in high water pH. Thus, at P_{CO_2} within the limits of pH_e compensation, water buffering capacity might be an important layer of habitat complexity that affects the performance and distribution of fishes regardless of whether they express $pH_{coupled}$ or pH_{pi}.

Our findings highlight an important role for water pH in determining the rate and degree of acute pH_e compensation in *P. hypophthalmus* specifically, and perhaps in fishes generally. This suggests hypercarbia-induced reductions in water pH may underlie previously unexplained reductions to the rate and degree of pH_e compensation in fishes expressing pH_{pi}. Based on these results, we suggest a higher limit for acute pH_e compensation closer to 3–4 kPa P_{CO_2} might be shared across teleosts when uninhibited by water pH. Low water buffering capacity might therefore be an important selective pressure for pH_{pi} at CO_2 tensions normally within the limits of acute pH_e compensation and $pH_{coupled}$.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.A.S., R.B.S., C.D., C.J.B., M.H., L.M.P.; Methodology: M.A.S., R.B.S., C.J.B.; Formal analysis: M.A.S., R.B.S., C.D., M.H., L.M.P.; Investigation: M.A.S., R.B.S., C.D., M.H., L.M.P.; Resources: T.W., M.B., D.T.T.H., N.T.P.; Writing - original draft: M.A.S., R.B.S.; Writing - review & editing: M.A.S.,

R.B.S., C.D., M.H., L.M.P., T.W., M.B., C.J.B.; Supervision: T.W., M.B., C.J.B.; Project administration: T.W., M.B., D.T.T.H., N.T.P., C.J.B.; Funding acquisition: T.W., M.B., D.T.T.H., N.T.P., C.J.B.

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