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

Michael A Sackville[1,*], Ryan B Shartau[1,*], Christian Damsgaard[2,3], Malthe Hvas[2], Le My Phuong[2], Tobias Wang[2], Mark Bayley[2], Do Thi Thanh Huong[3], Nguyen Thanh Phuong[3] and Colin J Brauner[1].

[1]Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4. [2]Department of Bioscience, Aarhus University, 8000 Aarhus-C, Denmark. [3]College of Aquaculture and Fisheries, Can Tho University, Can Tho, Vietnam.

*Authors contributed equally to this work

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Summary Statement

Low water pH limits extracellular pH compensation in a CO_2 tolerant fish. This may increase selection for a more robust CO_2 defense strategy where intracellular pH is preferentially regulated.

Abstract

Preferentially regulating intracellular pH (pH_i) confers exceptional CO_2 tolerance on fishes, but is often associated with reductions in extracellular pH (pH_e) compensation. It is unknown if 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 PCO_2 for 20 h at ecologically relevant water pH's 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 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.

Introduction

The aquatic partial pressure of carbon dioxide (PCO_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 PCO_2 exceed atmospheric levels by over 200-fold, and impose severe acute respiratory acidoses on fishes 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, 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 fishes use to compensate for acute respiratory acidoses (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, 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 trans-epithelial 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 intra- and extracellular compartments whether pH_e compensation occurs or not (Brauner and Baker, 2009; Occhipinti and Boron, 2015), and reductions to 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 PCO_2 in most freshwater fishes tested, while many fishes expressing pH_{pi} can robustly defend pH_i above 6 kPa PCO_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 mM required for full pH_e recovery at ~2 kPa PCO_2, let alone the ~100-150 mM 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 PCO_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.

Recently, the Mekong catfish *Pangasianodon hypophthalmus* was reported to fully compensate pH_e at 4 kPa PCO_2 (Damsgaard et al., 2015). Compensation was associated with a surprising ~45 mM 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 PCO_2. This would be in stark contrast with 19 of 20 CO_2 tolerant freshwater fishes tested (Shartau et al., 2016), including the Amazonian catfish *Pterygoplichthys pardalis,* which expresses pH_{pi} and negligible pH_e compensation at 1-6 kPa PCO_2 (Brauner et al. 2004). However, pH_i in the Mekong catfish *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 PCO_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 PCO_2 for 20 h in water artificially held at pH 4.5 or 5.8. Our results should provide further insight into the factors limiting $pH_{coupled}$ and selecting for pH_{pi}.

Materials and methods

Animal husbandry

Pangasianodon hypophthalmus were obtained from a local fish supplier in Can Tho, Vietnam and kept at Can Tho University for three months prior to experimentation. Fish were held in aerated 3000 L tanks fitted with a recirculating biofiltration system and kept on a 12:12 light:dark photoperiod. Water Cl^- and pH in these holding conditions were 0.35 mM and 7.2±0.1, respectively, which is similar to that listed for native habitat in the nearby Mekong River (in mM: [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 a commercial dry pellet obtained from a local supplier and held under these conditions for at least 3 weeks prior to experimentation. Fish wet mass ranged between 50-100 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) #A11-0235.

Protocol & 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 PCO_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. 4.5 was chosen as the lower water pH because it matches that of a previous study where the Amazonian catfish *Pterygoplichthys pardalis* was exposed to 3 kPa PCO_2 (Brauner et al., 2004). The desired PCO_2 and water pH for each treatment were reached within 15 minutes 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 PCO_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 PCO_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 constant water PCO_2 of 3 kPa (± 0.02 kPa) and full oxygen saturation. Fish were terminally sampled following 0, 3 and 20 h exposure to 3 kPa PCO_2 in both water pH treatments.

Prior to sampling, fish were rapidly transferred (<1-2 seconds) from experimental tanks by net to a neighboring 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 during euthanasia. 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 (pH_e). The spinal cord was then severed, and tissues (heart, liver and brain) were excised, wrapped in pre-labeled aluminum foil and frozen in liquid nitrogen. This entire procedure was completed within 2 minutes of ventilatory arrest. The second blood aliquot was centrifuged for three minutes at 6000 rpm to separate plasma and red blood cells (RBC's). Plasma and RBC's were frozen in liquid nitrogen with the tissue samples, and all samples were subsequently transferred to -80°C for storage until further analysis.

Extracellular, intracellular and water pH was measured with a Radiometer Analytical SAS pH electrode (GK2401C; Cedex, France) connected to a Radiometer PHM 84 (Copenhagen, Denmark) thermostatted to 28°C to match water temperature of the experiments. RBC pH_i was measured according to the freeze-thaw method (Zeilder 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 analyzer; Essex, UK). Blood PCO_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 analyzed 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 PCO_2 reduced pH_e from 7.79±0.02 to 7.40±0.03 and 7.45±0.012 in $pH5.8_{water}$ and $pH4.5_{water}$, 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 likely due to a net exchange of HCO_3^- and/or H^+ between the intra- and extracellular compartments, which is consistent with pH_{pi} expression (Heisler et al., 1982; Baker et al., 2009a).

After 20 h of hypercarbia, there was evidence for pH_e compensation in $pH5.8_{water}$ but little in $pH4.5_{water}$. In $pH5.8_{water}$, 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 mM at the respective PCO_2 (Fig. 1, P<0.01). In contrast, pH_e in $pH4.5_{water}$ 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 $pH5.8_{water}$ and $pH4.5_{water}$ (Fig. 2), but variation between tissues and treatments exists. 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 $pH5.8_{water}$ treatment (Fig. 2, P<0.05). Thus, brain pH_i appears more robustly defended than that of heart and liver, and heart and liver pH_i appears more tightly regulated in $pH4.5_{water}$ than

$pH5.8_{water}$. The latter difference could be attributed to a greater acidosis associated with higher in vivo PCO_2 in $pH5.8_{water}$ (Fig. 1), but this remains unknown. Red blood cell (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 $pH4.5_{water}$. Lack of RBC pH_i regulation is observed in all fishes expressing pH_{pi} to date (Shartau et al., 2016) and consistent with the absence of β-adrenergically stimulated NHE 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 PCO_2 normally within the limits of acute pH_e compensation and $pH_{coupled}$.

Impaired pH_e compensation in *P. hypophthalmus* at a water pH of 4.5 is associated with an absence of net trans-epithelial exchange of acid-base relevant ions. Low water pH is hypothesized to inhibit bicarbonate uptake and proton excretion by creating unfavourable trans-epithelial gradients for ion transport machinery (Parks et al., 2010) and/or directly impairing transporter structure-function (Kwong et al. 2014). Indeed, inhibition of trans-epithelial ion flux by low water pH at ambient PCO_2 has been shown in several fishes (Freda and McDonald, 1988; Shartau et al., 2017b; 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 if 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, 2017). 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 other ions on acid-base regulation in fishes.

Fishes expressing pH_{pi} often exhibit reduced rates and degrees of acute pH_e compensation relative to fishes expressing $pH_{coupled}$ (Shartau and Brauner, 2016). Furthermore, the approximate limit of 2 kPa PCO_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 a rate and degree of acute pH_e compensation in a freshwater fish expressing pH_{pi} that equals 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}/$pH_{coupled}$ or freshwater/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 PCO_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 PCO_2 in Vancouver city water, Canada; Shartau et al., 2017b), and both are lower than seawater (pH 6.9 at 3 kPa PCO_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 PCO_2 for acute pH_e compensation (Wood and LeMoigne, 1991; Baker and Brauner, 2009). However, rainbow trout exposed to hypercarbia in water at pH 6.9 fully compensated pH_e at ~3 kPa PCO_2 within 24-48 h (Dimberg, 1988; Larsen and Jensen, 1997). This was accomplished by a net 45 mM 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 PCO_2 across teleosts.

We are also first to observe pH_{pi} expression in the presence and absence of acute pH_e compensation at the same PCO_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 trans-epithelial 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 PCO_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 PCO_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 $pH_{coupled}$.

Competing interests

No competing interests declared.

Author contributions

MS and RS wrote the manuscript. All authors provided editorial input and contributed to experimental design, data collection and analysis.

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Figures

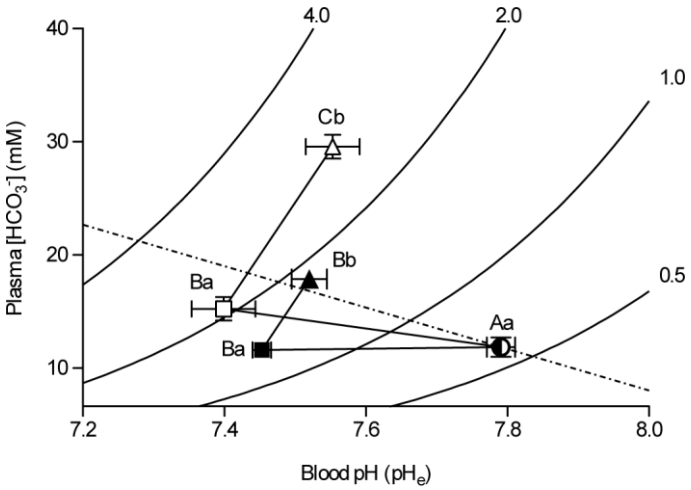

Fig. 1. Extracellular acid-base status in *Pangasianodon hypophthalmus* during exposure to 3 kPa PCO_2. Extracellular blood pH vs $[HCO_3^-]_{plasma}$ after 0 (circles), 3 (squares) and 20 h (triangles) exposure to 3 kPa PCO_2 at $pH4.5_{water}$ (shaded symbols) or $pH5.8_{water}$ (open symbols). Dashed and curved lines represent the blood non-bicarbonate buffer line and PCO_2-isopleths in kPa, respectively. Data presented as means±s.e.m. Upper and lower case letters indicate significant differences within treatments for blood pH and $[HCO_3^-]_{plasma}$, respectively (n=8, one-way ANOVA, $P<0.05$).

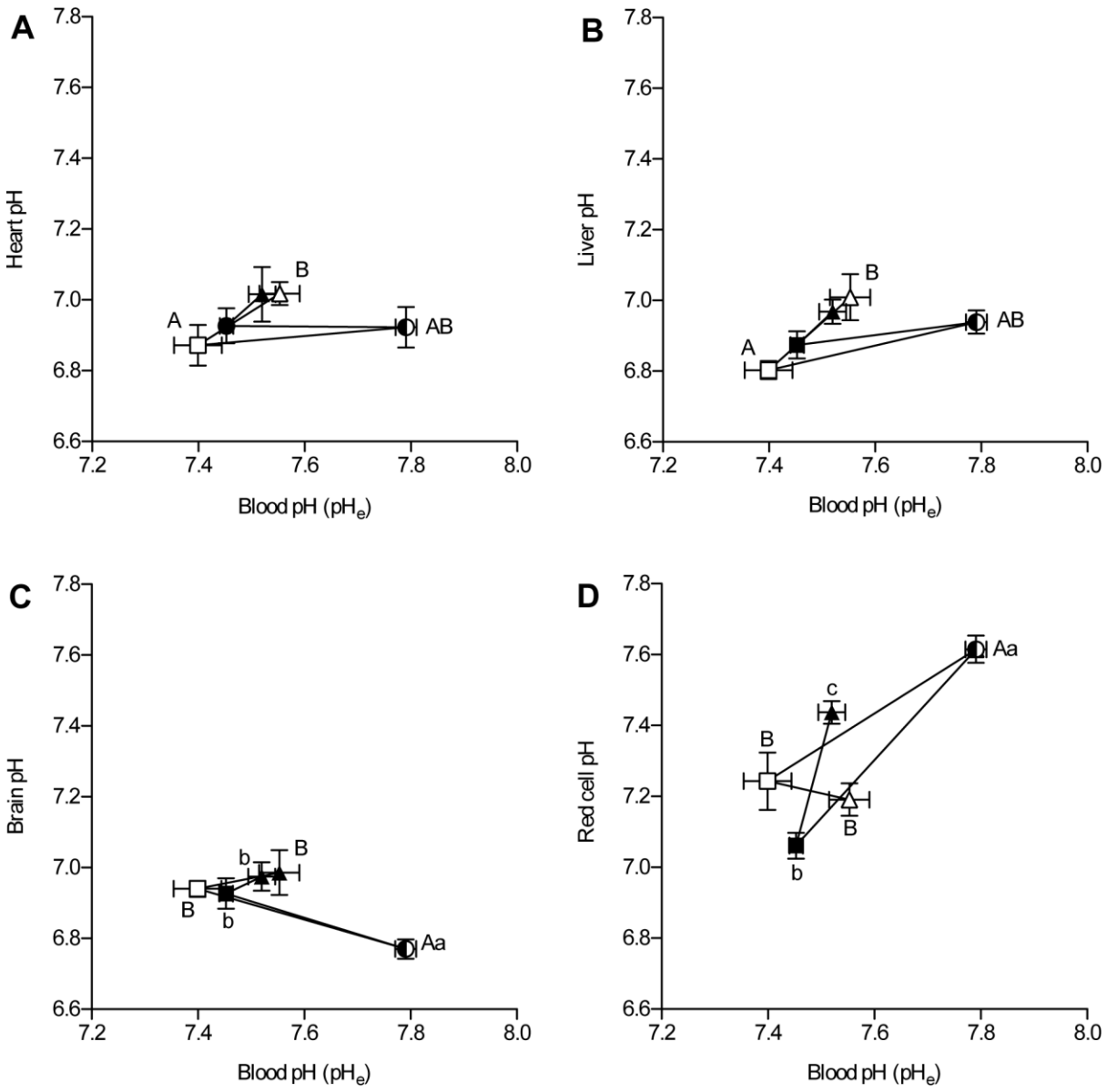

Fig. 2. Intracellular pH of *Pangasianodon hypophthalmus* during exposure to 3 kPa PCO_2. Extracellular blood pH vs intracellular pH of heart (A), liver (B), brain (C) and red blood cell (D) after 0 (circles), 3 (squares) and 20 h (triangles) exposure to 3 kPa PCO_2 at $pH4.5_{water}$ (shaded symbols) or $pH5.8_{water}$ (open symbols). Data presented as means±s.e.m. Lower and upper case letters indicate significant differences for intracellular pH in $pH4.5_{water}$ and $pH5.8_{water}$, respectively (n=8, one-way ANOVA, $P<0.05$).

Tables

Table 1. Plasma [Lactate$^-$] of *Pangasianodon hypophthalmus* after 0, 3 and 20 h in 3 kPa PCO_2 at water pH 5.8 or 4.5. Data presented as means±s.e.m. No significant differences from 0 h within treatments (n=8, one-way ANOVA, P>0.05).

	Plasma Lactate (mM)	
Time (h)	$pH5.8_{water}$	$pH4.5_{water}$
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