

SHORT COMMUNICATION

High capacity for extracellular acid–base regulation in the air-breathing fish *Pangasianodon hypophthalmus*

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ABSTRACT

The evolution of accessory air-breathing structures is typically associated with reduction of the gills, although branchial ion transport remains pivotal for acid–base and ion regulation. Therefore, air-breathing fishes are believed to have a low capacity for extracellular pH regulation during a respiratory acidosis. In the present study, we investigated acid–base regulation during hypercapnia in the air-breathing fish *Pangasianodon hypophthalmus* in normoxic and hypoxic water at 28–30°C. Contrary to previous studies, we show that this air-breathing fish has a pronounced ability to regulate extracellular pH (pH_e) during hypercapnia, with complete metabolic compensation of pH_e within 72 h of exposure to hypoxic hypercapnia with CO_2 levels above 34 mmHg. The high capacity for pH_e regulation relies on a pronounced ability to increase levels of HCO_3^- in the plasma. Our study illustrates the diversity in the physiology of air-breathing fishes, such that generalizations across phylogenies may be difficult.

KEY WORDS: Carbon dioxide, Hypercapnia, Hypoxia, Pangasius

INTRODUCTION

In most water-breathing fish species, respiratory acidosis (i.e. an acidosis caused by elevated CO_2) is compensated by transepithelial exchange of acid–base equivalents with the environment, i.e. excretion of H^+ or retention of HCO_3^- , such that extracellular pH (pH_e) is restored in the face of the increased partial pressure of carbon dioxide in the arterial blood (PaCO_2) (Claiborne et al., 2002; Evans et al., 2005; Perry and Gilmour, 2006). However, the gills of air-breathing fishes are generally reduced in size – an adaptation that is presumed to aid in avoiding branchial O_2 loss in hypoxic water – but have retained their ancestral function in acid–base and ion regulation (Graham, 1997; Tamura and Moriyama, 1976). It has therefore been suggested that the reduced surface area of the gills of air-breathing fishes places limitations on transepithelial ion exchange, thus constraining the branchial capacity for acid–base regulation (Brauner and Baker, 2009; Shartau and Brauner, 2014). Therefore, all studies on acid–base regulation in air-breathing fishes to date indicate a low capacity for exchange of acid–base equivalents in pH_e regulation and a preferential regulation of intracellular pH, during a respiratory acidosis (Brauner and Baker, 2009; Brauner et al., 2004; Harter et al., 2014b; Heisler, 1982; Shartau and Brauner, 2014).

Pangasianodon hypophthalmus is a facultative air-breathing fish, which uses a modified and highly vascularized swim bladder for air-breathing during environmental hypoxia (Lefevre et al., 2011a).

In contrast to some air-breathing fishes, *P. hypophthalmus* appears to have larger gills (Graham, 1997; Lefevre et al., 2011a; Tamura and Moriyama, 1976) that may allow for considerable branchial ion exchange, and thus confer high capacity for pH_e regulation during hypercapnia [increased water partial pressure of carbon dioxide (PwCO_2)]. *P. hypophthalmus* inhabits tropical freshwater plains within the Mekong River Delta in South-East Asia. Such ecosystems are characterized by frequent high organic loading resulting in combined hypoxia and hypercapnia with PwCO_2 reaching 65 mmHg (Furch and Junk, 1997; Ultsch, 1987; Willmer, 1934). Furthermore, it is farmed extensively in severely hypoxic (Lefevre et al., 2011b) and hypercapnic (this study) water throughout South-East Asia.

To investigate the ability of *P. hypophthalmus* to compensate pH_e during a respiratory acidosis, we measured pH_e , PaCO_2 and accumulation of HCO_3^- in cannulated fishes during exposure to two levels of hypercapnia (7 and 22 mmHg CO_2) in normoxic water at 28–30°C. In addition, we also investigated how *P. hypophthalmus* deals with the combined severe hypercapnia and hypoxia it experiences in natural tropical waters (Furch and Junk, 1997; Willmer, 1934) by exposing cannulated individuals to water circulated from a local aquaculture pond. We hypothesized that the large gills of *P. hypophthalmus* confer greater capacity to regulate pH_e during hypercapnia than observed in other air-breathing fishes with a more reduced gill size.

RESULTS AND DISCUSSION**Acid–base regulation during hypercapnia**

Within the first 3 h of each hypercapnia exposure, PaCO_2 rose and remained stable for the following 72 h (Fig. 1A). The initial rise in PaCO_2 was attended by a reduction in pH_e (Fig. 1B), while the calculated $[\text{HCO}_3^-]_{\text{plasma}}$ increased along the *in vitro* non-bicarbonate buffer line (β_{NB}) of -18.3 ± 5.2 slykes [i.e. pH_e fell with a slope of -16.5 ± 2.4 and -22.7 ± 4.1 slykes ($\text{mmol l}^{-1} \text{HCO}_3^- \text{ pH}_e^{-1}$) in the 22 mmHg CO_2 and pond treatments, respectively] (Fig. 2A). Hematocrit (Hct) was 15.3 ± 1.5 , 18.7 ± 2.6 , 17.2 ± 0.9 and 22.4 ± 1.6 % in fishes exposed to control, 7 mmHg, 22 mmHg and pond water, respectively, and fell slightly after 72 h to 13.9 ± 1.0 , 18.4 ± 4.2 , 13.2 ± 1.1 and 22.9 ± 3.5 % in the respective groups.

During continued hypercapnia, $[\text{HCO}_3^-]_{\text{plasma}}$ increased with a concurrent restoration of pH_e (Fig. 1B,C) along the PaCO_2 isopleths of the Davenport diagram (Fig. 2A), indicating that pH_e regulation is primarily metabolic involving a combination of HCO_3^- retention and/or H^+ excretion. There was a slight tendency for the PaCO_2 – PwCO_2 difference to decrease at higher PwCO_2 , possibly indicating an increased ABO efflux of CO_2 at increasing levels of hypercapnia, as also observed in South American lungfish (Sanchez et al., 2005). The rate of HCO_3^- accumulation was faster in fishes exposed to pond water, where the hypercapnia was most severe. pH_e was fully compensated within 24 h at 7 mmHg CO_2 and within 72 h at 22 mmHg CO_2 and exposure to pond water. This reveals a

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List of symbols and abbreviations

[CO ₂] _{total}	total plasma CO ₂ concentration
Hct	hematocrit
P _a CO ₂	arterial partial pressure of carbon dioxide
pH _e	plasma pH
pK	acid dissociation constant for CO ₂ hydration
P _w CO ₂	water partial pressure of carbon dioxide
P _w O ₂	water partial pressure of oxygen
α _{CO₂}	CO ₂ solubility in trout plasma
β _{NB}	non-bicarbonate buffer effect

pronounced capacity for metabolic pH_e regulation during hypercapnia in *P. hypophthalmus*. This is in stark contrast to all other air-breathing fishes studied to date that have a low capacity for pH_e regulation during a respiratory acidosis (Shartau and Brauner, 2014). Thus, the armored catfish *Liposarcus pardalis* only recovered 8% and 22% of the pH_e disturbance when exposed to ~7 mmHg CO₂ and 42 mmHg CO₂, respectively for 96 h (Brauner et al., 2004). Similarly, the bowfin *Amia calva* regulated 28% and 24% of the pH_e disturbance at 11 and 45 mmHg CO₂, respectively, after 24 h (Brauner and Baker, 2009) and *Arapaima gigas* only partly compensated the pH_e disturbance incurred after exposure to 40 mmHg CO₂ for 72 h (Gonzalez et al., 2010). No indication of extracellular acid–base regulation was detected in the South American lungfish (*Lepidosiren paradoxa*) over 50 h at 49 mmHg CO₂ (Sanchez et al., 2005). The ability to regulate pH_e during hypercapnia in *P. hypophthalmus* is also highly developed compared with several water-breathing fishes, such as the common carp *Cyprinus carpio*, which recovered 50% of the initial pH_e disturbance after 48 h exposure to 8 mmHg CO₂

(Claiborne and Heisler, 1984) and the white sturgeon *Acipenser transmontanus*, which only compensated 35% of pH_e after exposure to 28 mmHg for 72 h (Crocker and Cech, 1998). The European eel exhibits a medium capacity for acid–base regulation, compensating 75% of its disturbance after 6 weeks at a P_{CO₂} of 15 mmHg (McKenzie et al., 2003). *P. hypophthalmus* thus shows an acid–base regulatory capacity during a respiratory acidosis in line with active water-breathers such as cod and rainbow trout, which compensate the full pH_e disturbance within 24 h of exposure to 7.5 and 15 mmHg, respectively (Eddy et al., 1977; Larsen et al., 1997).

The metabolic compensation of pH_e in *P. hypophthalmus* includes an accumulation of plasma HCO₃[−] through HCO₃[−] retention and/or H⁺ excretion, which is generally thought to involve equimolar exchange of HCO₃[−] with Cl[−] and H⁺ with Na⁺, respectively (Claiborne et al., 2002; Evans et al., 2005; Perry and Gilmour, 2006). Here, [Cl[−]]_{plasma} decreased during all hypercapnia treatments (Fig. 1D) and individual reductions in [Cl[−]]_{plasma} were mirrored by increases in [HCO₃[−]]_{plasma}, suggesting involvement of a HCO₃[−]/Cl[−] exchanger in pH_e regulation during hypercapnia (Fig. 2B). However, in response to the most severe hypercapnia (34 mmHg CO₂), [HCO₃[−]]_{plasma} increased by approximately 30 mmol l^{−1} after 72 h, but was only accompanied by a ~12 mmol l^{−1} reduction in [Cl[−]]_{plasma}. This suggests that over this longer time course other mechanisms for ion exchange than an equimolar HCO₃[−]/Cl[−] anion exchanger are involved in pH_e regulation during hypercapnia. Alternatively, it has been proposed that freshwater fishes possess non-stoichiometric, HCO₃[−]/Cl[−] anion exchangers, exchanging multiple HCO₃[−]/Cl[−] ions, which might explain the non-stoichiometric changes in [HCO₃[−]]_{plasma} relative to [Cl[−]]_{plasma} (Grosell et al., 2009).

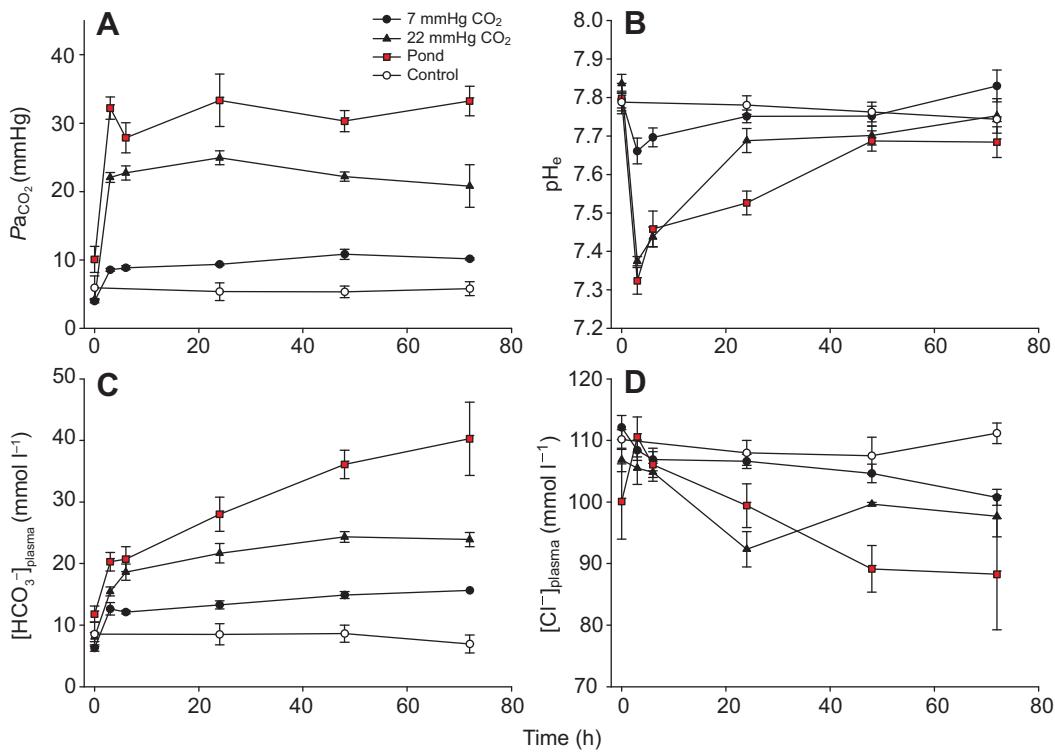


Fig. 1. Arterial plasma values in *Pangasianodon hypophthalmus* during exposure to hypercapnia. Arterial partial pressure of carbon dioxide (A), plasma pH (pH_e) (B), plasma [HCO₃[−]] (C) and plasma [Cl[−]] (D) in cannulated *P. hypophthalmus* during exposure to 7 mmHg CO₂, 22 mmHg CO₂, pond water (34 mmHg CO₂) and normocapnic water (control). Parameters at time zero were measured in well-aerated water just prior to exposure to experimental conditions. Data are means±s.e.m. (N=6).

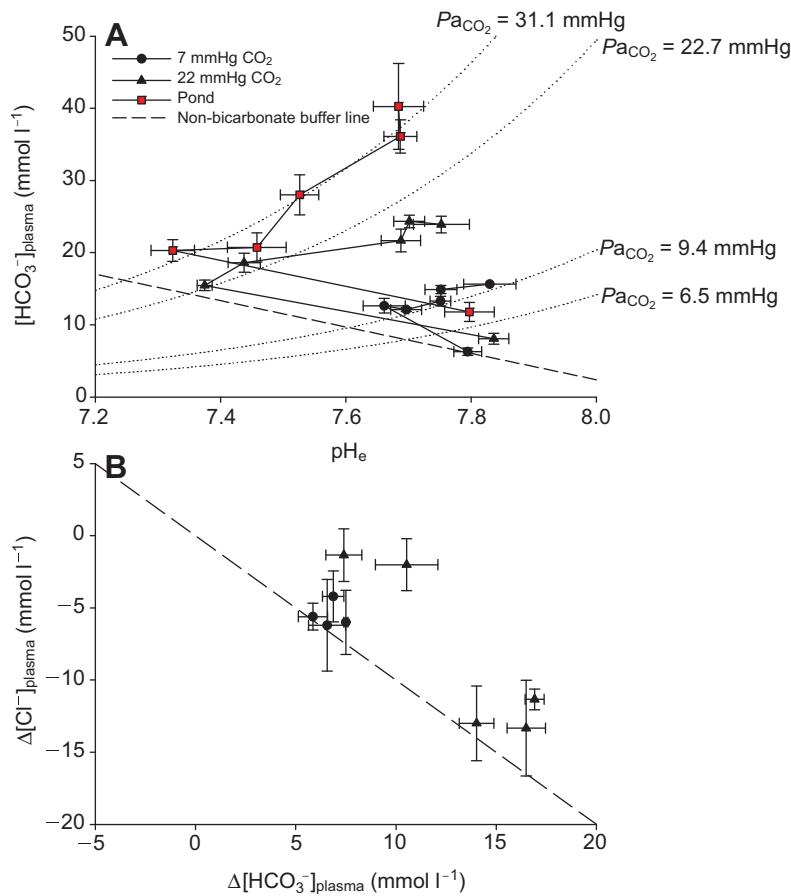


Fig. 2. Changes in acid-base parameters and $[Cl^-]_{\text{plasma}}$ during exposure to hypercapnia in cannulated *Pangasianodon hypophthalmus*. Fish were exposed to 7 mmHg CO_2 , 22 mmHg CO_2 and pond water and blood parameters were determined at 0, 3, 6, 24, 48 and 72 h. (A) Davenport diagram with CO_2 isopleths at P_{CO_2} upon exposure to 7 mmHg CO_2 , 22 mmHg CO_2 and pond water, respectively. Dashed line indicates non-bicarbonate buffer effect determined *in vitro*. (B) Individual changes in $[Cl^-]_{\text{plasma}}$ and $[HCO_3^-]_{\text{plasma}}$ after exposure to hypercapnia. Data are means \pm s.e.m. ($N=6$).

The metabolic acid–base compensation during hypercapnia seemed unaffected by hypoxia. During hypoxia, *P. hypophthalmus* decreases gill ventilation and the resulting reduced water flow might impose limitations on branchial ion exchange (Lefevre et al., 2011a). Thus, the acid–base regulation seen here either indicates a lack of limitation of ventilation or that there is a pronounced renal–intestinal ion exchange, which has also been suggested to be a general trait for air-breathing fishes (Graham, 1997; Perry and Gilmour, 2006).

Our study does not identify the anatomical, cellular or molecular components underlying the high capacity for pH_e regulation, but this might be associated with the large gills and hence ion-exchange surface area in *P. hypophthalmus* (Lefevre et al., 2011a). It might also be a consequence of a low HCO_3^-/Cl^- exchanger activity, augmenting HCO_3^- retention, which would be consistent with the proposed low Cl^- permeability of the gill of *P. hypophthalmus*, as indicated by a low uptake rate of nitrite (Lefevre et al., 2011c). Both characteristics contrast with those of other air-breathing fishes (Graham, 1997; Lefevre et al., 2014; Tamura and Moriyama, 1976). Other mechanisms may contribute to the high regulatory capacity for pH_e regulation in *P. hypophthalmus*, such as H^+/Na^+ exchange, greater renal or intestinal contribution in acid–base regulation, the ability to excrete CO_2 across the air-breathing organ etc.

We provide the first documentation of very high P_{wCO_2} in *P. hypophthalmus* aquaculture (supplementary material Fig. S1), and confirm previous studies of severe hypoxia in these ponds (Lefevre et al., 2011b). The severe hypercapnia observed here is in line with many other observations from naturally hypoxic tropical waters (Furch and Junk, 1997; Willmer, 1934) and exceeds the level of respiratory

acidosis that fishes are believed to be able fully to compensate for metabolically (Heisler, 1982; Larsen and Jensen, 1997; Crocker and Cech, 1998; Brauner et al., 2004). Under hypercapnic and hypoxic conditions (occurring frequently in both natural and aquaculture water in the tropics), *P. hypophthalmus* must live with an extensive metabolic compensation and a strongly elevated $[HCO_3^-]_{\text{plasma}}$ and thus suppressed Cl^- uptake. While the ability to regulate pH_e seems beneficial for tropical freshwater species, the physiological consequences of long-term maintenance of a pronounced metabolic compensation are unknown and should be a subject for future research.

In summary, the air-breathing *P. hypophthalmus* is endowed with ample capacity for pH_e regulation during hypercapnia, which contradicts the trend that air-breathing fishes are inefficient in extracellular acid–base regulation during a respiratory acidosis. The diversity of air-breathing fishes is considerable, with more than 400 species and 65 separate evolutionary events and it is therefore unsurprising that physiological challenges are accommodated using different physiological and anatomical building blocks in different groups. It may thus prove difficult to generalize on the capacity to cope with physiological challenges across the phylogenetically distinct air-breathing fishes.

MATERIALS AND METHODS

Animal handling

Pangasianodon hypophthalmus Sauvage 1878 were purchased from a local fish supplier and transferred to Can Tho University (Vietnam), where they were held in large well-aerated tanks at 27°C for 4 months prior to experimentation. The fishes were fed daily with commercially purchased dry pellets and water was changed every third day. All experiments were performed in accordance with national guidelines for the protection of animal welfare in Vietnam.

Experimental protocols

Series I: acid–base regulation during normoxia

A total of 22 fishes (mass, 831 ± 66 g; length, 43 ± 1 cm; means \pm s.e.m.) were anaesthetized in 0.1 g l^{-1} benzocaine and a polyethylene PE50 catheter was inserted into the dorsal aorta through the dorsal side of the mouth (Soivio et al., 1975) while the gills were irrigated with well-oxygenated water with 0.05 g l^{-1} benzocaine. After recovery for ~ 24 h in well-aerated water, a normoxic blood sample was taken and the fishes were exposed to normoxic water ($P_{O_2}\sim 120\text{ mmHg}$) at $28\text{--}30^\circ\text{C}$ containing either 7 mmHg or 22 mmHg CO_2 , supplied from a 500 l recirculating tank. P_{wCO_2} was monitored using an Oxyguard Pacific system coupled with a G10ps CO_2 probe and a K01svpld pH probe (Oxyguard International A/S, Farum, Denmark), which supplied CO_2 to the water when pH increased above a value corresponding to the desired P_{wCO_2} . At each CO_2 level, 1 ml blood was sampled from the catheter for immediate determination of blood gases at 0, 3, 6, 24, 48 and 72 h. P_{aCO_2} and pH_e were measured using a handheld iStat with a G3+ cartridge (Abbot Point of Care Inc., Princeton, NJ, USA). Hct was measured as the fractional volume of red blood cells in blood after centrifugation at 12,000 r.p.m. for 3 min. Total plasma CO_2 concentration ($[CO_2]_{\text{plasma}}$) was measured according to Cameron, 1971. Hemoglobin (Hb) concentration was measured spectrophotometrically after conversion to metHb. $[Cl^-]_{\text{plasma}}$ was measured using a chloride titrator (Sherwood model 926S MK II Chloride analyzer). The concentration of NH_3 in the water was measured using the phenol–hypochlorite method; water NO_2^- was measured using the Griess reaction and water NO_3^- levels were measured using the salicylate method.

Series II: acid–base regulation during hypoxia

Twelve fishes (mass= 1152 ± 56 g; length= 45 ± 0.7 cm) with arterial catheters were allowed to recover for ~ 24 h in well-aerated water and then exposed to water from a Vietnamese aquaculture pond pumped from 2 m depth (pond was 3.5 m deep). A 300 μl blood sample was taken from each fish through the catheter at 0, 3, 6, 24, 48 and 72 h for immediate determination of P_{aCO_2} , pH_e , Hct, [Hb] and $[Cl^-]_{\text{plasma}}$. Pond water conditions were: $[NH_3]=0.058\text{ }\mu\text{mol l}^{-1}$; $[NO_2^-]=1.9\text{ }\mu\text{mol l}^{-1}$; $[NO_3^-]=1.3\text{ }\mu\text{mol l}^{-1}$; $pH=6.29$; $\text{Temp}=30.8\pm0.4$; $P_{wO_2}=16.8\pm13.4\text{ mmHg}$; $P_{wCO_2}=34.3\pm3.3$ (means \pm s.d.).

Blood tonometry: non-bicarbonate buffering and calibration of iStat P_{aCO_2}

The effect of β_{NB} was found by equilibrating 5 ml blood from 11 individual fishes in an Eschweiler tonometer with humidified gas mixtures provided from two serial-linked gas mixing pumps (Wösthoff, Bochum, Germany). Oxygen-saturated blood was equilibrated with 7 and 22 mmHg CO_2 for at least 30 min to allow for full equilibration of the blood with the gas. $[CO_2]_{\text{total}}$ was measured as described above and β_{NB} was calculated as $\Delta[HCO_3^-]_{\text{total}} \times \Delta pHe^{-1}$ assuming a linear relationship (Nightingale and Fedde, 1972). Prior to the experiments, all gas mixing pumps were validated using a Servomex 570A Oxygen Analyzer.

Harter et al. (2014a) recently criticized the reliability of the iStat for blood gas measurements in fish at 10 and 20°C . To validate our iSTAT measurements from blood at 30°C , it was necessary to check for errors associated with the problems in temperature compensation (Malte et al., 2014). Thus, we measured P_{CO_2} of blood samples equilibrated to 7, 22 and 37 mmHg CO_2 in tonometers receiving water saturated gas mixtures delivered by Wösthoff pumps. This direct comparison revealed a slight, but consistent underestimation of P_{CO_2} by the iStat compared with true P_{CO_2} delivered by the Wösthoff pumps (supplementary material Fig. S2A). We corrected all measurements of P_{aCO_2} according to the regression presented in supplementary material Fig. S2A.

Calculations

Blood acid dissociation constant for CO_2 hydration (pK) was calculated from the Henderson–Hasselbach equation:

$$pK = pH_e - \log\left(\frac{[HCO_3^-]_{\text{plasma}}}{\alpha_{CO_2} P_{aCO_2}}\right). \quad (1)$$

In Series I and in blood tonometry, $[HCO_3^-]_{\text{plasma}}$ was calculated by subtracting physically dissolved CO_2 from $[CO_2]_{\text{total}}$ using temperature-

compensated CO_2 solubility in trout plasma (α_{CO_2}) from Boutilier et al. (1985).

In Series II, $[HCO_3^-]_{\text{plasma}}$ was calculated from Eqn 1 (supplementary material Fig. S2B) using P_{aCO_2} and pH_e from iStat, pK ($pK=4.83+0.17\text{ pH}_e$) from Series I and α_{CO_2} from Boutilier et al. (1985).

Partial pressures of oxygen and carbon dioxide in aquaculture ponds

P_{wCO_2} , P_{wO_2} , pH_w and temperature were measured in 12 Vietnamese aquaculture ponds at 0, 1, 2 and 3 meters depth with either small (30–50 g) or large fishes (400–1000 g) using an Oxyguard Pacific Commander box fitted with a pH and P_{CO_2} probe and an YSI ProODO optical dissolved oxygen meter (YSI Inc., Yellow Springs, OH, USA). P_{wCO_2} at the surface was significantly higher in ponds with large fish ($18.0\pm1.8\text{ mmHg}$ and $2.9\pm0.8\text{ mmHg}$, respectively; $1\text{ mmHg}=133\text{ Pa}$; supplementary material Fig. S1A) and increased with water depth ($P_{wCO_2}=18.3\text{ mmHg}+2.76\text{ mmHg m}^{-1}$; $F_{1,19}=4.53$, $P<0.05$, $R^2=0.15$). pH_w at the surface was correspondingly lower in ponds with large fish compared with small fish (6.37 ± 0.03 and 7.10 ± 0.11 , respectively; supplementary material Fig. S1B). The corresponding P_{wO_2} was higher in ponds with small fish compared with large fish (108 ± 13 and $44\pm13\text{ mmHg}$, respectively; supplementary material Fig. S1C) and decreased significantly with depth in ponds with small fish ($P_{wO_2}=109\text{ mmHg}-16.3\text{ mmHg m}^{-1}$; $F_{1,20}=7.408$, $P<0.05$, $R^2=0.27$).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.D., L.T.H.G., D.D.T. and P.V.T. performed experiments; C.D. analyzed data and prepared figures; C.D., T.W. and M.B. designed the study, interpreted results and edited, revised and drafted manuscript; all authors approved final version of manuscript.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.117671/-DC1>

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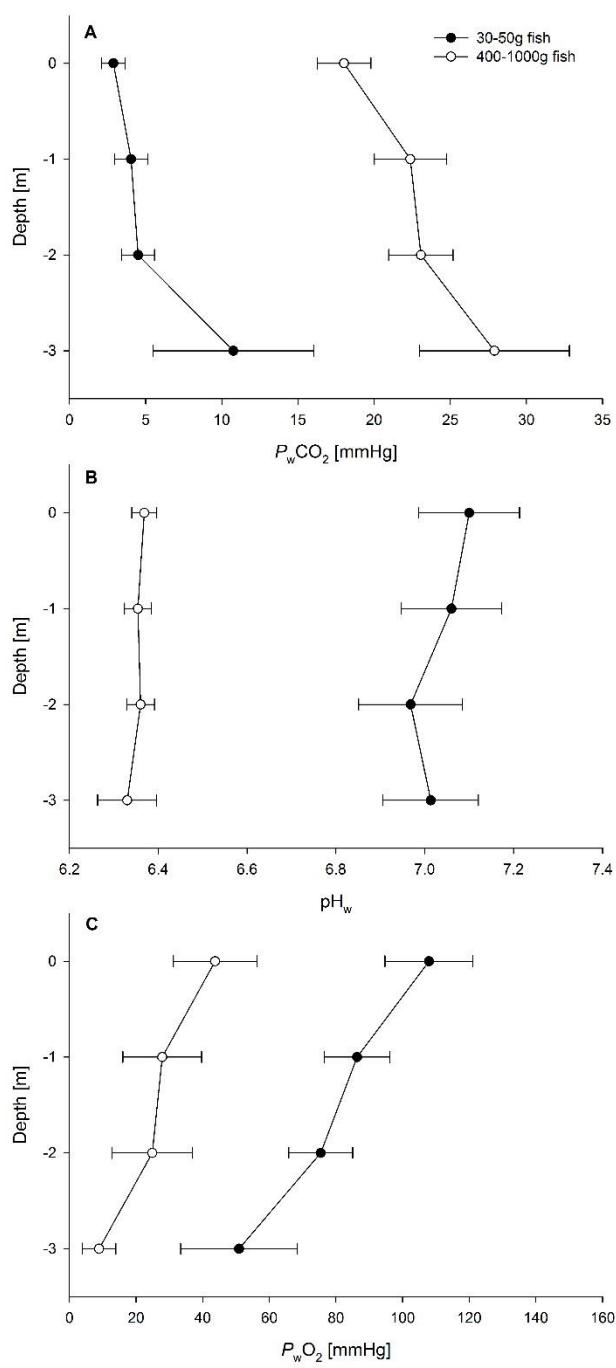


Fig. S1. Physical and chemical conditions of Vietnamese *Pangasius* aquaculture ponds. Water partial pressure of carbon dioxide ($P_w\text{CO}_2$) (A), pH (pH_w) (B) and partial pressure of oxygen ($P_w\text{O}_2$) (C) at different depths of Vietnamese *Pangasianodon hypophthalmus* aquaculture ponds with 30-50 g fish (filled circles) and with 400-1000 g fish (empty circles). Data are means \pm s.e.m. ($n=6$).

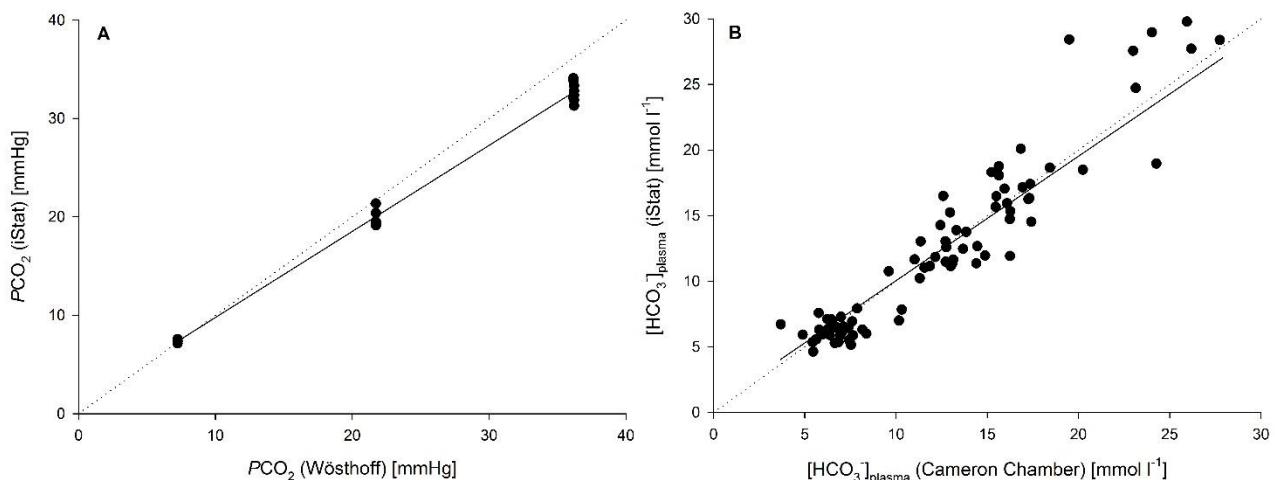


Fig. S2.Verification of PCO_2 and $[\text{HCO}_3^-]$ from iStat. A: Correlation between true P_{CO_2} in a blood sample and the P_{CO_2} measured by the iStat. Equation for linear least squares regression is $P_{\text{aCO}_2} (\text{true}) = 1.03 + 0.875 P_{\text{aCO}_2} (\text{iStat})$, $F_{1,16}=2992$, $P<0.001$, $r^2 = 0.99$. Solid lines indicate the line for linear least squares regression and dotted lines indicate line of identity. B: Correlation between $[\text{HCO}_3^-]_{\text{plasma}}$ measured using the method from (Cameron, 1971) and using the Henderson Hasselbach equation applying $p\text{H}_e$ and P_{aCO_2} from iStat, temperature compensated α_{CO_2} from (Boutilier et al., 1985) and $p\text{H}_e$ compensated pK . Equation for linear least squares regression is $[\text{HCO}_3^-]_{\text{plasma}} (\text{iStat}) = 0.56 + 0.95 [\text{HCO}_3^-]_{\text{plasma}} (\text{Cameron Chamber})$, $F_{1,81}=476$, $P<0.001$, $r^2=0.85$.