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ORIGINAL PAPER

Preferential intracellular pH regulation represents a general pattern of pH homeostasis during acid–base disturbances in the armoured catfish, *Pterygoplichthys pardalis*

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Abstract Preferential intracellular pH (pH_i) regulation, where pH_i is tightly regulated in the face of a blood acidosis, has been observed in a few species of fish, but only during elevated blood PCO_2. To determine whether preferential pH_i regulation may represent a general pattern for acid–base regulation during other pH disturbances we challenged the armoured catfish, *Pterygoplichthys pardalis,* with anoxia and exhaustive exercise, to induce a metabolic acidosis, and bicarbonate injections to induce a metabolic alkalosis. Fish were terminally sampled 2–3 h following the respective treatments and extracellular blood pH, pH_i of red blood cells (RBC), brain, heart, liver and white muscle, and plasma lactate and total CO_2 were measured. All treatments resulted in significant changes in extracellular pH and RBC pH_i that likely cover a large portion of the pH tolerance limits of this species (pH 7.15–7.86). In all tissues other than RBC, pH_i remained tightly regulated and did not differ significantly from control values, with the exception of a decrease in white muscle pH_i after anoxia and an increase in liver pH_i following a metabolic alkalosis. Thus preferential pH_i regulation appears to be a general pattern for acid–base homeostasis in the armoured catfish and may be a common response in Amazonian fishes.

Keywords Acidosis · Alkalosis · Amazon · Armoured catfish · Intracellular pH regulation

Abbreviations

Cl^-	Chloride
CO_2	Carbon dioxide
DO	Dissolved oxygen
Hb	Haemoglobin
Hct	Haematocrit
N_2	Nitrogen
$NaHCO_3^-$	Sodium bicarbonate
O_2	Oxygen
PCO_2	Partial pressure of CO_2
pH_e	Extracellular pH
pH_i	Intracellular pH
PO_2	Partial pressure of O_2
RBC	Red blood cell
TCO_2	Total CO_2
WM	White muscle

Introduction

The regulation of acid–base status is a fundamental process associated with homeostasis in living organisms. The conformation of many proteins, and hence enzymes, is sensitive to changes in pH (e.g. Hazel et al. 1978) and thus organismal functional integrity depends upon tight

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regulation of pH at the extracellular and cellular level. Acid–base regulation in fish has been studied for decades, although only in a few species of teleosts and elasmobranchs (see Brauner and Baker 2009). Many studies have used short-term (up to 96 h) hypercarbia (elevated environmental PCO_2) to induce a respiratory acidosis, and the common response in water breathers is a net increase in plasma HCO_3^- in exchange for Cl^-, mediated predominantly through branchial processes (Heisler 1984; Claiborne et al. 2002; Evans et al. 2005). Most fish species studied to date cannot fully compensate for a respiratory acidosis associated with PCO_2 levels above 15 mmHg (Brauner and Baker 2009). At increasing levels of hypercarbia, complete recovery of extracellular pH (pH_e) appears to be limited by some characteristics of net plasma HCO_3^-/Cl^- exchange, termed the "bicarbonate concentration threshold" (Heisler 1984). The latter likely determines CO_2 tolerance and ultimately survival in hypercarbia exposed fish (Brauner and Baker 2009).

During a respiratory acidosis, changes in blood pH_e are associated with qualitatively similar, albeit smaller, changes to intracellular pH (pH_i) (Wood and LeMoigne 1991; Wood et al. 1990). This is typically the result of a higher intrinsic buffering capacity of the intracellular compartment and/or active regulation of pH_i. In many teleosts red blood cell (RBC) pH_i is regulated by β-adrenergically stimulated sodium proton exchangers (β-NHE) that are activated during stress (Nikinmaa 1990). However, all siluriformes, including armoured catfish, lack β-NHEs and H^+ distribution across the RBC membrane is largely passive (Val et al. 1998; Berenbrink et al. 2005). In these animals, RBC pH_i is protected only by their high buffering capacity; the result of a high intracellular haemoglobin concentration (Wood and LeMoigne 1991). Despite this exception, most tissues in vertebrates have some capacity to actively regulate pH_i (Madshus 1988; Putnam and Roos 1997), but this capacity is limited and most tissues regulate pH_i in proportion to pH_e during recovery from a respiratory acidosis (Heisler 1984; Brauner and Baker 2009; Wood et al. 1990; Wood and LeMoigne 1991). Consequently, most fish investigated are incapable of complete protection of pH_i, beyond the "bicarbonate concentration threshold" (associated with PCO_2 ~ 15 mmHg) and without at least partial recovery of blood pH_e (Claiborne and Heisler 1986; Pörtner et al. 1998; McKenzie et al. 2003).

Remarkably, some of the most CO_2 tolerant fishes studied to date tightly regulate the pH_i of vital tissues during a hypercarbia-induced acidosis, while pH_e remains largely uncompensated. This protective strategy, termed preferential pH_i regulation, deviates dramatically from the pattern of pH regulation commonly accepted for vertebrates (Brauner and Baker 2009; Brauner et al. 2004). Preferential pH_i regulation was first documented in the salamander, *Siren lacertina* (Heisler et al. 1982) and in the facultative air breathing teleost *Synbranchus marmoratus* (Heisler 1982), two species that were able to protect pH_i of the heart and white muscle (WM) despite a large uncompensated reduction in blood pH_e during a respiratory acidosis. More recently, a third air-breathing species, the armoured catfish, *Pterygoplichthys pardalis* (formerly known as *Liposarcus pardalis*) was added to this group. When exposed to environmental hypercarbia (up to 42 mmHg PCO_2) pH_e in the armoured catfish decreased severely (from pH 7.98 to values around pH 6.99) and remained largely uncompensated within the following 96 h (Brauner et al. 2004). Despite this general acidosis, the pH_i of brain, heart, liver and WM remained unchanged. *P. pardalis* is one of the most CO_2 and acidosis-tolerant teleost species examined to date (Brauner et al. 2004); thus it has been proposed that preferential pH_i regulation is associated with CO_2 tolerance in fish (Brauner and Baker 2009). This hypothesis is further supported by a non-air-breathing fish species, the white sturgeon (*Acipenser transmontanus*), which was found to preferentially regulate pH_i of WM, heart, brain and liver, even under prolonged (48 h) exposure to severe hypercarbia (45 mmHg PCO_2) (Baker et al. 2009a). Subsequent research has improved our understanding of preferential pH_i regulation (Baker et al. 2009b; Baker et al. 2011; Huynh et al. 2011; Hanson et al. 2009); however, all current studies used hypercarbia to induce acid–base disturbances. It remains unclear if preferential pH_i regulation is a unique response to elevated PCO_2 or the associated respiratory acidosis. We hypothesise that animals capable of preferentially regulating pH_i during hypercarbia employ this strategy as a general pattern of pH homeostasis.

To test this hypothesis, the present study investigated the regulatory response of *P. pardalis* following acid–base disturbances other than a respiratory acidosis induced by hypercarbia. Therefore, animals were challenged with (1) anoxia (metabolic acidosis originating in all tissues), (2) repeated exhaustive exercise (metabolic acidosis originating in WM) or (3) injection of an iso-osmotic bicarbonate solution (metabolic alkalosis originating in the blood). Sampling was 2–3 h following the respective treatments, to ensure sufficient time for all compartments to experience the acid–base disturbance. The acid–base status of the animals was assessed in the extracellular compartment as whole blood pH_e and in the intracellular compartment by measuring the pH_i of RBC, brain, heart, liver and WM. In addition, lactate levels were measured in the plasma and bony tissue collected from acidosis-exposed animals. Previously, it has been proposed that *P. pardalis* may incorporate lactate into their large bony structures during an acidosis (MacCormack et al. 2006), a strategy that has been described in turtles and caimans (Jackson 1997; Jackson et al. 2000; Jackson et al. 2003). We believe this work

will provide insight into whether preferential pH_i regulation in *P. pardalis* is strictly a response to a hypercarbia-induced, respiratory acidosis or whether it represents a general strategy for pH homeostasis over a range of acid–base disturbances.

Materials and methods

Animals and housing

Experiments were performed during two separate research stays (2004 and 2013) at the Brazilian National Institute for Research in the Amazon (INPA), Manaus, Brazil. Armoured catfish, *Pterygoplichthys pardalis (Castelnau 1855)* (228 ± 21 g) were caught in the Rio Negro near Manaus using gill nets. Animals were held at INPA on a natural photoperiod in outdoor tanks for at least 2 weeks prior to experimentation and without being fed. Fish were held in air saturated well-water at 28 °C and pH of 6.9 ± 0.3 (see Brauner et al. 2004 for additional water composition). All experiments were performed in compliance with the local legislation on laboratory animal care (CEUA-INPA 047/2012).

Experimental protocol

The day prior to experiments, fish were retrieved from the holding tank, weighed and then housed individually overnight in 12 l tanks, supplied with moderate water flow (to ensure water turnover every 30 min) from a common recirculating system. Even though animals had access to the surface at all time, no air-breathing behaviour was observed during acclimation, treatments or recovery. Within a series, individual tanks were randomly assigned to either a control or a treatment group and experiments were carried out the next day. Treatment animals were challenged with either (1) anoxia, (2) repeated exhaustive exercise or (3) injection of an iso-osmotic bicarbonate solution. Since pH_e disturbances are not immediately transferred to the tissues, animals were allowed to recover for 2–3 h before terminal sampling (Brauner et al. 2004).

Series 1: acidosis induced by exposure to aquatic anoxia ($n = 6$)

Treatment tanks were continuously bubbled with N_2 and dissolved oxygen (DO) levels in the water were monitored with an YSI 550A DO meter (Yellow Springs, OH, USA). Within 5 min, DO levels decreased below 0.2 mg l^{-1}. Throughout the trial N_2 flow was kept constant and DO levels remained below detection limit; control tanks were aerated with ambient air. Fish were exposed to anoxia or control for 1 h and were then allowed to recover for 3 h before terminal sampling, during which time all tanks were aerated with ambient air to achieve 100 % air saturation.

Series 2: acidosis induced by exhaustive exercise ($n = 9$)

Fish were removed from tanks and subjected to a repeated exhaustive exercise protocol in a 20 l chamber, where fish were chased by prodding with a piece of soft rubber tubing for 20 min. This procedure was repeated a second time 2 h after the first exercise event and fish were allowed to recover for a 2 h period before sampling; control fish remained undisturbed until sampling.

Series 3: alkalosis induced by sodium bicarbonate ($NaHCO_3^-$) injection into the caudal vein ($n = 6$)

All tanks were disconnected from the recirculation system and animals were lightly anaesthetised in their tanks with 0.1 g l^{-1} Tricaine methanesulfonate (MS-222) buffered with $NaHCO_3^-$. After 2 min fish were injected into the caudal vein with a mass specific volume of a 140 mM $NaHCO_3^-$ solution (in distilled water) or an equimolar sodium chloride (NaCl) solution as a sham control. The handling time and air exposure during this procedure was typically less than 20 s. The injected volumes were chosen to initially triple the plasma $[HCO_3^-]$ (in the $NaHCO_3^-$ treatment) based upon the following assumptions: in the armoured catfish soft tissue comprises 60 % of the total body mass (lower than in most teleosts due to the large bony structures of the skull and on the skin); 5 % of the soft tissue weight is blood; and the blood plasma of control fish has a $[HCO_3^-]$ of approximately 9 mM (Brauner et al. 2004). Injecting 0.15 ml per ml of blood (or 0.45 ml per 100 g of animal body weight) of a 140 mM $NaHCO_3^-$ solution was expected to increase plasma $[HCO_3^-]$ from 9 mM to about 27 mM, assuming that all HCO_3^- remained in the plasma. Following the injections, fish were placed back into their tanks and allowed to recover for 3 h before sampling.

Terminal sampling

Fish were terminally sampled according to Brauner et al. (2004), where fish were anaesthetised in their housing tanks by mixing in a concentrated solution of buffered MS-222 to achieve a final concentration of 0.5 g l^{-1}. After about 3–5 min fish were unresponsive but still ventilating. Blood (0.5 ml) from the caudal vein was drawn into a heparinized syringe and stored on ice for analysis. The spinal cord was severed and brain, heart, liver, and WM were rapidly removed by dissecting the animals (within 2 min). Tissue samples were immediately frozen in liquid N_2 and

stored at −80 °C for analysis. Bony material was collected from the skull, pectoral fins, spine and scales. These samples were ground to a fine powder under liquid N_2 using a freezer mill (Certiprep Spex 6700, Metuchen, NJ, USA) and dried at 104 °C overnight. Lactate samples were taken from animals of the anoxia treatment and control (*series 1*) and from animals of a separate exercise treatment following the same protocol as for *series 2,* where the induced blood acidosis was not significantly different from values reported in *series 2* ($p > 0.05$).

Analysis

Intra- (pH_i), extracellular (pH_e) and water pH measurements were performed using a Radiometer PHM 84 (Copenhagen, Denmark) connected to a Radiometer Analytical SAS pH electrode (GK2401C, Cedex, France) thermostated at 28 °C. RBC pH_i was measured according to the freeze–thaw method of Zeidler and Kim (1977) and tissue pH_i was measured according to Pörtner et al. (1990) and Baker et al. (2009b).

Haematocrit (Hct) was measured in duplicate after centrifugation at 12,000 rpm for 3 min. Haemoglobin concentration [Hb] was measured spectrophotometrically by converting all Hb into cyanomethaemoglobin and using an extinction coefficient of 11. Plasma was obtained by centrifuging whole blood at 6,000 rpm for 3 min. Total CO_2 (TCO_2) in the blood plasma was measured with a Corning 965 CO_2 analyzer (Essex, UK). Plasma [HCO_3^-] was calculated from the plasma pH_e and TCO_2 using the rearranged Henderson-Hasselbalch equation. The CO_2 solubility coefficient and pK for plasma were determined from Boutilier et al. (1984). Bone lactate levels were measured according to Jackson et al. (2003), where aliquots of dry powder were incubated at room temperature in 0.73 mol l^{-1} trichloroacetic acid (10 ml g^{-1}). [Lactate] was measured in the bone solutions and in plasma using the lactic oxidase/peroxidase colorimetric assay (Sigma Procedure 735) at 540 nm using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK).

Statistics

All data were analysed with the IBM SPSS Statistics v20 software (IBM SPSS, Chicago, IL, USA). Homogeneity of variances was tested with the Levene's test ($p < 0.05$) and normality of distribution was tested with the Shapiro–Wilkinson test ($p < 0.05$). Hct data were log-transformed and animal weight data were $1/(x + 1)$ transformed to achieve normality of distribution. Differences between control and treatment group means were compared using an Independent samples *t*-test ($p < 0.05$; *series 1,* $n = 6$; *series 2,* $n = 9$; *series 3,* $n = 6$; unless indicated otherwise). Control group means between series were analysed by One-way ANOVA (Tukey, $p < 0.05$). All data are presented as mean ± s.e.m.

Table 1 Animal weight, haematocrit (Hct) and haemoglobin concentration [Hb] of whole blood in armoured catfish 2–3 h after exposure to anoxia (*series 1*), exhaustive exercise (*series 2*) or a metabolic alkalosis (*series 3*)

	N		Weight (g)	Hct (%)	[Hb] (mM)
Series 1:	6	Control	379 ± 16^a	28 ± 1^a	1.20 ± 0.04^a
		Anoxia	431 ± 28	38 ± 2*	1.45 ± 0.07*
Series 2:	9	Control	156 ± 18^b	22 ± 2^a	1.18 ± 0.16^a
		Exercise	128 ± 4	29 ± 2	1.30 ± 0.12
Series 3:	6	Control	170 ± 60^b	25 ± 4^a	1.38 ± 0.19^a
		Alkalosis	189 ± 27	28 ± 4	1.52 ± 0.14

Data represent mean ± s.e.m

* Indicates a statistically significant difference between the treatment and control mean, within the same series (Independent sample *t*-test, $p < 0.05$)

Superscript letters that differ indicate statistically significant differences among control means between series (One-way ANOVA, Tukey, $p < 0.05$)

Results

Control group animal weights were significantly ($p < 0.05$) higher in *series 1*, compared to *series 2* and *3* which did not differ ($p > 0.05$, Table 1). Animal weights between controls and treatments of the same series were not significantly different ($p > 0.05$). No significant differences were detected in Hct and [Hb] control means between series ($p > 0.05$). After anoxia exposure (*series 1*) Hct increased significantly by 10 ± 3 % and [Hb] increased by 0.25 ± 0.12 mM ($p < 0.05$). No changes in Hct or [Hb] were observed after exercise or $NaHCO_3^-$ injections (*series 2* and *3, p* > 0.05).

Extracellular acid–base status

Whole blood pH (pH_e) was not significantly different between series control groups and on average was 7.69 ± 0.02 ($p < 0.05$; Fig. 1a). In *series 1*, aquatic anoxia resulted in a significant reduction in pH_e by 0.50 ± 0.09 pH units ($p < 0.05$). In *series 2*, exhaustive exercise significantly reduced pH_e by 0.25 ± 0.04 pH units ($p < 0.05$). In *series 3*, $NaHCO_3^-$ injection into the caudal vein significantly increased pH_e by 0.16 ± 0.03 pH units ($p < 0.05$).

Plasma TCO_2 was significantly ($p < 0.05$) lower in control fish of *series 1* compared to both *series 2* and *3*, which were not significantly different from each other ($p > 0.05$) and on average were 10.49 ± 0.33 mM. In *series 1*, anoxia led to a significant decrease in plasma TCO_2, by 3.50 ± 0.65 mM, from 6.51 ± 0.62 to 3.01 ± 0.65 mM

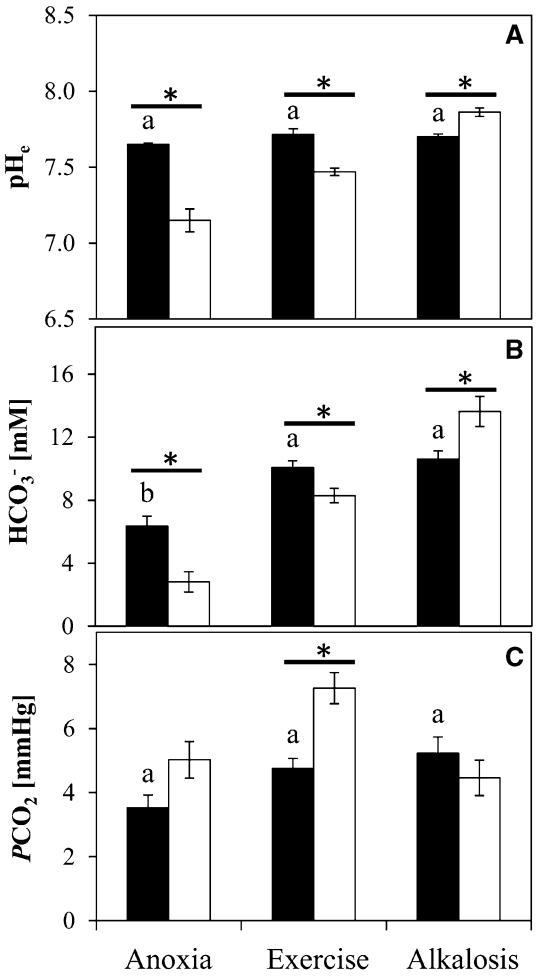

Fig. 1 **a** Extracellular pH (pH_e), **b** plasma HCO_3^- concentration (mM) and **c** PCO_2 (mmHg), of armoured catfish, 2–3 h after exposure to anoxia (*series 1*, $n = 6$), exhaustive exercise (*series 2*, $n = 9$) or a metabolic alkalosis (*series 3*, $n = 6$). *Black and white bars* are control and treatment values, respectively, presented as mean ± s.e.m. Superscript letters that differ indicate statistically significant differences between control means (One-way ANOVA, Tukey, $p < 0.05$) and *Asterisk* indicates a statistically significant difference between treatment and control means within one series (Independent sample *t*-test, $p < 0.05$)

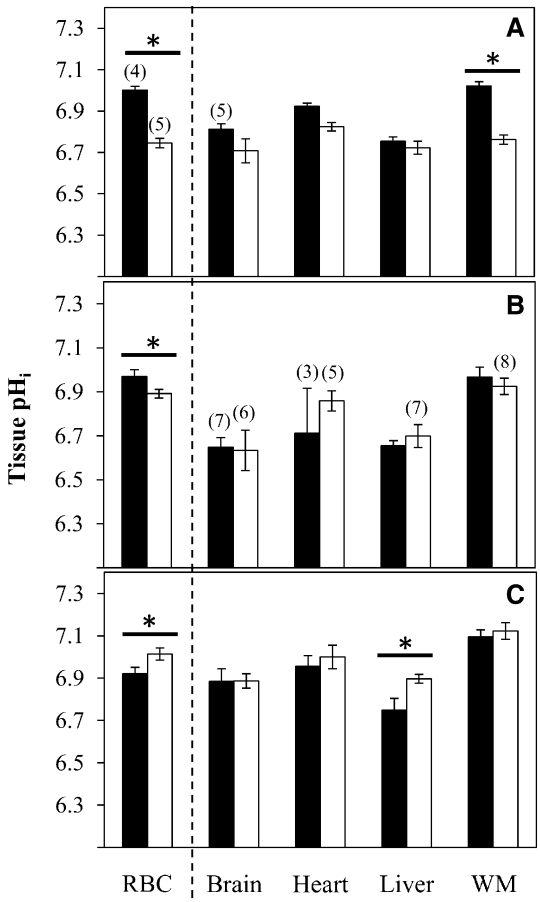

Fig. 2 Intracellular pH (pH_i) of red blood cells (RBC), brain, heart, liver and white muscle (WM) tissue of armoured catfish, 2–3 h after exposure to **a** anoxia (*series 1*, $n = 6$), **b** exhaustive exercise (*series 2*, $n = 9$) or **c** a metabolic alkalosis (*series 3*, $n = 6$). *Black and white bars* are control and treatment values, respectively, presented as mean ± s.e.m (pH_e mean ± s.e.m. are as reported in Fig. 1a). *Bars* are annotated with their respective *n*-values, if different from above and *Asterisk* indicates a statistically significant difference between treatment and control means within one tissue (Independent sample *t*-test, $p < 0.05$)

($p < 0.05$). In *series 2*, repeated exhaustive exercise caused a significant decrease in plasma TCO_2, by 1.69 ± 0.48 mM, from 10.3 ± 0.4 to 8.6 ± 0.5 mM ($p < 0.05$). In *series 3*, injection of $NaHCO_3^-$ into the caudal vein caused a significant increase in plasma TCO_2, by 2.99 ± 0.99 mM, from 10.8 ± 0.5 to 13.8 ± 1.0 mM ($p < 0.05$).

Plasma [HCO_3^-] was significantly lower in control fish of *series 1* compared to both *series 2* and *series 3*, which were not significantly different from each other ($p > 0.05$) and on average were 10.30 ± 0.33 mM (Fig. 1b). Exposure to anoxia (*series 1*) and exhaustive exercise (*series 2*) resulted in a significant reduction in plasma [HCO_3^-] by 3.56 ± 0.64 mM and 1.79 ± 0.48 mM, respectively ($p < 0.05$). The induced metabolic alkalosis (*series 3*) resulted in a significant increase in plasma [HCO_3^-] by 3.02 ± 0.97 mM ($p < 0.05$).

Plasma PCO_2 of control fish was not significantly different between treatments and on average was 4.53 ± 0.26 mmHg ($p > 0.05$, Fig. 1c). Exhaustive exercise (*series 2*) caused a significant increase in PCO_2, by 2.51 ± 0.47 mmHg ($p < 0.05$); but neither anoxia ($p = 0.056$) nor the metabolic acidosis had a significant effect on plasma PCO_2 ($p > 0.05$).

Intracellular acid–base status

Series 1: exposure to aquatic anoxia led to a significant decrease in RBC pH_i by 0.26 ± 0.03 pH units and WM pH_i by 0.26 ± 0.03 pH units (Fig. 2a, $p < 0.05$). No significant

Table 2 Lactate levels of blood plasma and bony structures in armoured catfish 2–3 h after exposure to an anoxia- or exercise-induced acidosis

	N	Plasma (μmol ml^{-1})	Skull (μmol g^{-1})^{A}	Pectoral (μmol g^{-1})^{A}	Spine (μmol g^{-1})^{A}	Scales (μmol g^{-1})^{A}
Control	6	0.5 ± 0.1^{a}	6.6 ± 1.3^{a}	7.9 ± 1.3^{a}	6.4 ± 0.5^{a}	9.0 ± 1.3^{a}
Anoxia	6	9.3 ± 1.3^{b}	5.1 ± 0.6^{a}	7.4 ± 1.6^{a}	4.9 ± 1.4^{a}	5.9 ± 0.3^{a}
Exercise	5	6.3 ± 0.7^{b}	5.2 ± 0.6^{a}	6.5 ± 0.5^{a}	6.5 ± 1.9^{a}	9.7 ± 2.4^{a}

Data represent mean ± s.e.m

[A] Lactate levels expressed based on wet mass

Superscript letters that differ indicate statistically significant differences between series (One-way ANOVA, Tukey, $p < 0.05$)

differences were observed between control and treatment pH_i of brain, heart or liver tissue ($p > 0.05$) and average pH_i was 6.76 ± 0.04, 6.87 ± 0.02 and 6.74 ± 0.02, respectively.

Series 2: following repeated exhaustive exercise and 2 h of recovery, only RBC pH_i decreased significantly by 0.08 ± 0.04 pH units ($p < 0.05$, Fig. 2b). No significant differences were observed between control and treatment pH_i of brain, heart, liver or WM tissue ($p > 0.05$) and average pH_i was 6.64 ± 0.05, 6.82 ± 0.06, 6.68 ± 0.03 and 6.95 ± 0.03, respectively.

Series 3: a mass specific injection of $NaHCO_3^-$ into the caudal vein and 3 h of recovery led to a significant increase in RBC pH_i by 0.09 ± 0.03 pH units and liver pH_i by 0.15 ± 0.06 pH units ($p < 0.05$, Fig. 2c). No significant differences were observed between control and treatment pH_i of brain, heart or WM tissue ($p > 0.05$) and average pH_i was 6.89 ± 0.03, 6.98 ± 0.04, 7.11 ± 0.03, respectively.

Lactate incorporation into the bone and skin following anoxia or exercise

Exposure to a metabolic acidosis in both anoxia-exposed (*series 1*) and exercised fish (*series 2*), caused a significant increase ($p < 0.05$) in plasma [lactate], compared to control values (Table 2). However, neither treatment led to a significant accumulation of lactate in the skull, pectoral-, spine-bones nor scales of armoured catfish ($p > 0.05$).

Discussion

The aim of the present study was to determine whether the armoured catfish employ preferential pH_i regulation as a general strategy of acid–base regulation, regardless of the origin of the induced pH disturbance. By challenging animals with a metabolic acidosis induced by anoxia or exhaustive exercise, as well as an alkalosis induced by $NaHCO_3^-$ injections, we provide support for the hypothesis that armoured catfish tightly regulate tissue pH_i in response to all types of episodic acid or base loading events. Furthermore, this strategy of pH regulation was maintained during pH challenges that span the range of blood pH_e values likely to be experienced *in vivo* (pH 7.15–7.86).

Extracellular acid–base status

Exposure to anoxia in *series 1* led to an anticipated, significant decrease ($p < 0.05$) in whole blood pH_e of nearly 0.5 pH units compared to control fish (Fig. 1a). Severe hypoxia has been described to induce acidoses of similar magnitude in other species, for example rainbow trout (Thomas et al. 1986) or the hypoxia-tolerant Amazonian cichlid *Astronotus ocellatus* (Richards et al. 2007). When subjected to repeated exhaustive exercise in *series 2*, whole blood pH_e decreased significantly ($p < 0.05$) by 0.25 pH units compared to resting control fish (Fig. 1a). This is in line with previous work on exercise in rainbow trout (Milligan and Wood 1986) and the facultative air-breathers bowfin (*Amia calva*) (Gonzalez et al. 2001) and spotted gar (*Lepisosteus oculatus*) (Burleson et al. 1998). In *series 3*, the injection of armoured catfish with an iso-osmotic $NaHCO_3^-$ solution led to a significant ($p < 0.05$) increase in whole blood pH_e by 0.16 pH units, compared to sham-injected control fish (Fig. 1a). Despite the additional handling and air exposure during injections, control fish pH_e in *series 3* was not significantly ($p < 0.05$) different from control values in *series 1* or *2*. Consequently, a super-imposed acidosis induced by handling, which could have masked the metabolic alkalosis, was likely negligible. Other studies that have injected fish with 140 mM $NaHCO_3^-$ solutions have found larger changes to pH_e compared to the present study (Goss and Wood 1990; Goss and Perry 1994; Goss et al. 1994; Gilmour et al. 2011). However, these studies employed continuous infusion protocols instead of single injections and comparisons to the present results are therefore, difficult to interpret.

Intracellular acid–base status

RBCs in the armoured catfish lack active pH_i control and have the highest intrinsic buffering capacity of the studied tissues (Wood and LeMoigne 1991). Therefore, changes in RBC pH_i are indicative of a sufficiently severe pH_e

disturbance that will likely be reflected in pH_i changes of any other tissue lacking active pH_i regulation (Brauner et al. 2004; Baker et al. 2009a). As expected, the induced changes to blood pH_e led to a corresponding and significant ($p < 0.05$) change in RBC pH_i in all three series. This suggests that the induced acid–base disturbances and the chosen sampling points were adequate to affect the pH_i of other unregulated tissues. Remarkably, and in agreement with previous work (Brauner et al. 2004), the pH_i of the sampled tissues remained tightly regulated during both metabolic acidoses, induced by anoxia and exhaustive exercise (*series 1* and *2*) and the metabolic alkalosis induced by $NaHCO_3^-$ injections ($p > 0.05$, Fig. 2), indicating that pH_i regulation in these tissues was up-regulated in response to the induced acid–base disturbances. Interestingly, the pH_i of liver tissue increased significantly ($p > 0.05$) after $NaHCO_3^-$ injection, by approximately 0.15 pH units. Since changes in liver metabolism or composition were not assessed, the reason for this increase in pH_i remains unknown and is worthy of further study.

A metabolic acidosis in fish exposed to anoxia or after exercise is the result of both ATP hydrolysis and regeneration through the anaerobic glycolytic pathway, which produces H^+ and lactate (Robergs et al. 2004). Due to the high glycolytic capacity of WM in fish, this tissue is presumably the largest source of H^+ during both anoxia and exercise. This was reflected in a significant ($p < 0.05$) reduction in WM pH_i following anoxia, but surprisingly not following exhaustive exercise. While the present data do not allow any definitive conclusions on this discrepancy, it can be speculated that the capacity for exercise in *P. pardalis* (a sluggish, bottom-dwelling detritivore) and the resulting acidosis was simply not large enough to significantly alter the pH_i of WM tissue. H^+ that are produced during glycolysis are buffered by HCO_3^- within the cells and the blood plasma, resulting in the formation of CO_2, elevating PCO_2 and reducing plasma [HCO_3^-] (Beaver et al. 1986; Wasserman et al. 1967; Turrell and Robinson 1942). This was reflected in the present results, where plasma PCO_2 increased significantly after exercise ($p < 0.05$, Fig. 1c) and possibly after anoxia exposure (whereas significant only at the $p = 0.056$ level); both treatments led to a significant decrease in plasma [HCO_3^-] ($p < 0.05$, Fig. 1b).

In addition to a severe metabolic acidosis, the accumulation of lactate from anaerobic metabolism may represent a limiting factor for the ability of fish to cope with extended hypoxia or exhaustive exercise. Both turtles (Jackson et al. 2000) and caimans (Jackson et al. 2003) prolong survival during hypoxia by relocating lactate from the bloodstream to bony tissue. The capacity of armoured catfish to use their bony structures (which make up 40 % of their body mass) to buffer lactate has not been fully investigated. MacCormack et al. (2006) found equivocal indications that lactate levels increased in the skull of *P. pardalis* following approximately 3 h of anoxia exposure. Our findings do not support this conclusion. Despite a significant increase in plasma [lactate] of fish exposed to anoxia or exhaustively exercised, no significant accumulation of lactate was measured in skull, pectoral-, spine bones or scales. Therefore, buffering of lactate by bony structures does not seem to be part of the mechanism by which armoured catfish cope with short-term exposure to anoxia or exercise. This does not exclude, however, the involvement of bony structures in lactate buffering during prolonged anoxia exposure or chronic stress (MacCormack et al. 2006).

The present data suggest that, in line with our previous hypothesis, preferential pH_i regulation in *P. pardalis* is not strictly a response to a hypercarbia induced, respiratory acidosis. The pH_i of vital tissues remained tightly regulated following metabolic acidoses and alkalosis, despite an incomplete recovery of pH_e. While in the present study only short-term responses to acid–base disturbances were assessed, Brauner et al. (2004) reported that even after 96 h of continuous exposure to a severe respiratory acidosis (42 mmHg PCO_2), tissue pH_i remained protected while blood pH_e recovery was limited to just over 20 %. Therefore, it seems that preferential pH_i regulation in *P. pardalis* cannot only be sustained for extended periods of time, but may in fact represent the predominant strategy for acid–base homeostasis in this species.

So why don't *P. pardalis* rely on pH_e regulation to a greater extent?

Perhaps revealing is that all fish species studied to date that exhibit preferential pH_i regulation (*S. marmoratus*, *P. pardalis* and *A. transmontanus*) are endemic to highly dilute freshwater environments. Baker et al. (2009a), who first described preferential pH_i regulation in white sturgeon, reported an ionic water composition of: [Na^+] and [Cl^-] <3 μmol l^{-1}. These values are lower than those of the Amazonian waters used in the present study, albeit in a similar range: [Na^+], 15; [Cl^-], 16 μmol l^{-1} (Brauner et al. 2004). Fish species that are endemic to dilute waters typically have high-affinity transporters and/or decreased ion efflux rates, to maintain homeostasis under these conditions (Wood et al. 2002; Gonzalez and Wilson 2001; Preest et al. 2005). The functional relationship between ionoregulation and acid–base regulation in fish is still poorly understood and whether different strategies for ionoregulation in dilute waters correlate with the adopted strategy for acid–base regulation is an exciting avenue for future research. At the gills, the recovery of pH_e from an acidosis may involve active uptake of HCO_3^- in exchange for Cl^- or active extrusion

of H^+ in exchange for Na^+ (or a combination of both); however, the specific mechanisms involved remain largely unknown (Evans et al. 2005; Perry and Gilmour 2006). In either instance, the availability of counter-ions between the environment and the blood, or between the blood and the tissues will influence the rate and degree of pH recovery (Larsen and Jensen 1997; Brauner and Baker 2009). Regardless, ions are more abundant in the plasma compared to any freshwater environment, which may represent the inherent advantage of preferential pH_i regulation. Another advantage is that Cl^- transferred from the tissues to the plasma (in exchange for HCO_3^-) during pH_i regulation is not immediately lost to the environment (as would be the case during pH_e recovery) and thus, Cl^- shifts are restricted within the animal. Lastly, because tissue pH_i is typically lower compared to pH_e (−0.5 pH units) and is closer to the equilibrium constant ($pK = 6.4$) for the hydration of CO_2, smaller changes in $[HCO_3^-]$ are required intracellularly to correct for a given acidosis (Baker et al. 2009a). Therefore, preferential pH_i regulation may be particularly advantageous if branchial uptake of HCO_3^- is limited, and the available HCO_3^--pool is used most efficiently to safeguard the pH_i of vital tissues.

The Amazon River is characterised by high temperatures, stagnant waters, a high biological oxygen demand and often extensive vegetation cover. Especially during the dry season, resident fish species must cope with severe bouts of both hypoxia and hypercarbia (Brauner et al. 2004; Heisler 1984). Recent findings on a broader range of Amazonian fish species suggest that in this habitat, preferential pH_i regulation may be a relatively common strategy (unpublished data RS, DB, TH, CB, AV). Extending hypercarbia tolerance beyond the limits dictated by the "bicarbonate concentration threshold" and maintaining Cl^- (and/or Na^+) homeostasis in an ion-poor environment may represent strong selective pressures for the evolution of preferential pH_i regulation in fishes. Therefore, it is not surprising that some of the most hypoxia- and hypercarbia-tolerant teleost species inhabit Amazonian waters and that these traits are correlated with the occurrence of preferential pH_i regulation as an alternative strategy for pH homeostasis. In addition, most recent research provides putative evidence for preferential pH_i regulation in spotted gar, longnose gar (*Lepisosteus osseus*), alligator gar (*Atractosteus spatula*), bowfin and South American lungfish (*Lepidosiren paradoxa*) (Shartau and Brauner 2013), all of which are air-breathing fish species (as are *P. pardalis* and *S. marmoratus*). This is in line with Brauner and Baker (2009), who proposed that preferential pH_i regulation may also be a common pattern of acid–base regulation in bimodal fishes, a topic clearly in need of further study.

Conclusion

In the present study armoured catfish, *P. pardalis*, were subjected to pH disturbances, presumably spanning a large portion of their *in vivo* pH tolerance. During the induced metabolic acidoses, as well as a metabolic alkalosis, the pH_i of vital tissues remained tightly regulated, while blood pH_e regulation was incomplete. Interestingly, liver pH_i was regulated during the imposed acidoses, yet increased significantly during exposure to a metabolic alkalosis. Measurements of plasma and bony tissue [lactate] suggest that unlike turtles and caimans, armoured catfish do not buffer plasma lactate in bony structures. Preferential pH_i regulation appears to represent a general pattern for acid–base regulation regardless of the origin of the pH disturbance in armoured catfish and may be a relatively common pattern of acid–base regulation in Amazonian fishes and among bimodal fishes, an area ripe for further investigation.

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