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# Limited extracellular but complete intracellular acid–base regulation during short-term environmental hypercapnia in the armoured catfish, *Liposarcus pardalis*

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## Summary

**Environmental hypercapnia induces a respiratory acidosis that is usually compensated within 24–96 h in freshwater fish. Water ionic composition has a large influence on both the rate and degree of pH recovery during hypercapnia. Waters of the Amazon are characteristically dilute in ions, which may have consequences for acid–base regulation during environmental hypercapnia in endemic fishes. The armoured catfish *Liposarcus pardalis*, from the Amazon, was exposed to a water $P_{CO_2}$ of 7, 14 or 42 mmHg in soft water (in μmol $l^{-1}$: $Na^+$, 15, $Cl^-$, 16, $K^+$, 9, $Ca^{2+}$, 9, $Mg^{2+}$, 2). Blood pH fell within 2 h from a normocapnic value of 7.90±0.03 to 7.56±0.04, 7.34±0.05 and 6.99±0.02, respectively. Only minor extracellular pH ($pH_e$) recovery was observed in the subsequent 24–96 h. Despite the pronounced extracellular acidosis, intracellular pH ($pH_i$) of the heart, liver and white muscle was tightly regulated within 6 h (the earliest time at which these parameters were measured) *via* a rapid accumulation of intracellular $HCO_3^-$. While most fish regulate $pH_i$ during exposure to environmental hypercapnia, the time course for this is usually similar to that for $pH_e$ regulation. The degree of extracellular acidosis tolerated by *L. pardalis*, and the ability to regulate $pH_i$ in the face of an extracellular acidosis, are the greatest reported to date in a teleost fish. The preferential regulation of $pH_i$ in the face of a largely uncompensated extracellular acidosis in *L. pardalis* is rare among vertebrates, and it is not known whether this is associated with the ability to air-breathe and tolerate aerial exposure, or living in water dilute in counter ions, or with other environmental or evolutionary selective pressures. The ubiquity of this strategy among Amazonian fishes and the mechanisms employed by *L. pardalis* are clearly worthy of further study.**

Key words: acid–base regulation, hypercapnia, $pH_e$, $pH_i$, intracellular pH regulation, catfish, *Liposarcus pardalis*, Amazon, water hardness, air-breathing.

## Introduction

Environmental hypercapnia is common in freshwater systems with the most extreme examples being tropical aquatic environments covered with dense mats of vegetation, where $CO_2$ tensions can rise to as high as 60 mmHg (1 mmHg= approx. 0.133 kPa; Heisler et al., 1982). Hypercapnia causes a respiratory acidosis that, in most fish, is corrected within 10–72 h through branchial transfer of acid–base relevant ions (Heisler, 1984), while the kidney plays a relatively minor role (Perry et al., 1987). As a result, plasma $HCO_3^-$ levels are elevated and plasma $Cl^-$ levels reduced, implicating gill $Cl^-/HCO_3^-$ exchange as the primary mechanism for extracellular pH ($pH_e$) compensation. In general, intracellular pH ($pH_i$) compensation follows a qualitatively similar temporal pattern (Heisler, 1984; Cameron and Kormanik, 1982).

Complete or significant degrees of pH compensation during environmental hypercapnia have been demonstrated in numerous species of freshwater and marine fish (e.g. Toews et al., 1983; Claiborne and Heisler, 1984; Cameron, 1985; Goss et al., 1995; Larsen and Jensen, 1997; Larsen et al., 1997; reviewed by Heisler, 1984; Claiborne et al., 2002). Water composition clearly affects the rate of acid–base compensation where the rate and degree of compensation is more pronounced in seawater than in freshwater, and more pronounced in hard

compared to soft freshwater (Heisler, 1984; Larsen and Jensen, 1997).

Many natural systems are characterized by soft water. One example is the Amazon, where the ionic composition of many rivers and tributaries approaches that of distilled water (Val and Almeida-Val, 1995). Given that extensive vegetation covers many of the stagnant lakes and ponds in the Amazon during the dry season, it is likely that resident fishes experience periods of hypercapnia (Heisler et al., 1982). The ability of fishes endemic to these ion-poor waters to tolerate and compensate for environmental hypercapnia has not previously been investigated. The armoured catfish *Liposarcus pardalis* is common in the Amazon and can withstand relatively severe environmental hypercapnia and large disturbances in plasma pH (Randall et al., 1996). Here, we investigate the temporal regulation of extra- and intracellular acid–base status during environmental hypercapnia of *L. pardalis* in natural softwater. The first experimental series describes extracellular acid–base status during hypercapnia ($P_{CO_2}$ of 7, 14 and 42 mmHg) in softwater, while the second series investigates intracellular acid–base parameters of heart, liver and muscle during hypercapnia ($P_{CO_2}$ of 14 and 32 mmHg). The third experimental series investigates whole animal fluxes of $Na^+$, $Cl^-$ and ammonia during hypercapnia ($P_{CO_2}$ of 14 and 32 mmHg) to elucidate the mechanisms for acid–base regulation.

## Materials and methods

### *Animal acquisition and holding*

*Liposarcus pardalis* (Castelnau 1855) (308±15.5 g) were obtained from a commercial fish supplier and held at the National Institute for Research of the Amazon (INPA), Manaus, Brazil, for at least 2 weeks prior to experimentation. Fish were maintained in aerated well-water in outdoor tanks on a natural photoperiod. The ionic composition (in μmol $l^{-1}$) was $Na^+$, 15; $Cl^-$, 16; $K^+$, 9; $Ca^{2+}$, 9; $Mg^{2+}$, 2. Water temperature was 28°C, and water pH was 6.9±0.3. Fish were fed commercially purchased pellets on a maintenance ration of 1% body mass per day, but feeding was withheld 48–72 h prior to experimentation.

### *Experimental protocols*

#### *Series I: Effect of hypercapnia on extracellular acid–base status*

Fish were anaesthetized in 0.1 g $l^{-1}$ Tricaine Metanesulfonate (MS-222) buffered with $NaHCO_3^-$. Once anaesthetized, fish were transferred to a surgery table, and an indwelling catheter (PE-50) was placed in the dorsal aorta according to Soivio et al. (1975). Fish were left to recover for 24–48 h in individual chambers, and the cannulae were flushed several times with heparinized saline. Following recovery, the fish were placed in 2 l chambers without access to air and exposed to a $P_{CO_2}$ of 7, 14 or 42 mmHg. The low levels were achieved using a flow meter that mixed $CO_2$ with air into a 500 l recirculating system, while the high level was achieved using flow-through well-water directly from its source without prior aeration. Water $CO_2$ levels stabilized within 1 h. Blood samples were withdrawn from the cannulae at 0, 2, 6 and 24 h at each level of hypercapnia. Whole blood pH ($pH_e$), $P_{O_2}$, true plasma total $CO_2$ content, haemoglobin concentration ([Hb]), and haematocrit (Hct) were measured immediately, and plasma was frozen for later analysis of $Na^+$, $Cl^-$ and protein concentrations (see Analytical procedures below). Cannulated fish exposed to 42 mmHg did not survive for 24 h. Thus, non-cannulated fish were exposed to 42 mmHg for 96 h, and a blood sample was obtained from the caudal vein within 10 s of removal from the water.

#### *Series II: Effect of hypercapnia on intracellular acid–base status*

Non-cannulated fish were placed in individual 2 l chambers overnight before being exposed to a $\dot{P}_{CO_2}$ of 14 or 32 mmHg without access to air. Tissues were sampled from normocapnic control fish and at 6, 24 or 72 h into the hypercapnic period. For tissue sampling, water flow to the chamber was stopped and a concentrated solution of buffered MS-222 was slowly added to the water to achieve a final concentration of 0.1 g $l^{-1}$, according to Wang et al. (1994). Within 2–3 min, fish lost equilibrium and could be removed from the water without struggling. This procedure has been shown to minimize any metabolic and acid–base changes associated with handling and sampling. Blood (0.5 ml) was drawn from the caudal vein into a heparinized syringe for measurement of $pH_e$, red cell intracellular pH ($pH_i$), $P_{CO_2}$, [Hb], Hct and plasma $Na^+$, $Cl^-$, $Ca^{2+}$ concentrations, and osmolarity. The fish were then killed, and 0.5 g samples from heart, liver and muscle were removed and frozen immediately in liquid nitrogen for later analysis of $pH_i$. Sampling was complete within 2–3 min after the fish had been removed from the water.

#### *Series III: Effect of hypercapnia on whole animal ion fluxes*

Non-cannulated fish were placed in individual chambers (500 ml) overnight and exposed to the same regime as fishes of Series II. Fish were subjected to a $P_{CO_2}$ of 14 or 32 mmHg. At 0, 6 and 24 h (and at 72 h at $P_{CO_2}$ of 32 mmHg), water flow was interrupted for 1 h for measurements of whole animal unidirectional $Na^+$ influx ($J_{in}^{Na}$), efflux ($J_{out}^{Na}$), net flux ($J_{net}^{Na}$), net $Cl^-$ flux ($J_{net}^{Cl}$), and total ammonia excretion.

### *Analytical procedures*

Blood $P_{O_2}$ and $P_{CO_2}$ values were measured using Radiometer (Copenhagen, Denmark) $P_{O_2}$ (E5046) and $P_{CO_2}$ (E5036) electrodes thermostatted at 28°C in a BMS Mk2 electrode assembly with the output displayed on a Radiometer PHM 73. Total $CO_2$ content of true plasma was measured according to Cameron (1971). Extra- and intracellular pH were measured using a Radiometer microcapillary electrode (G299A) held within the BMS Mk2 system. Red cell $pH_i$ was measured using the freeze–thaw method of Zeidler and Kim (1977), and $pH_i$ of heart, liver and white muscle was measured according to Pörtner et al. (1990). Water pH was measured using a Corning (Corning, USA) combination pH electrode.

Haematocrit was measured in duplicate after centrifuging blood in micro-haematocrit tubes at 12 000 r.p.m. for 3 min. Blood [Hb] was measured spectrophotometrically following conversion to cyanomethaemoglobin, applying a millimolar extinction coefficient of 11.0. Plasma $Na^+$ and $Ca^{2+}$ levels were measured using atomic absorption flame photometry, and plasma $Cl^-$ concentration was measured according to Zall et al. (1956). Plasma ammonia, urea, glucose and lactate were measured using Sigma (St Louis, USA) diagnostic kits, and gill homogenate $Na^+$,$K^+$-ATPase activity was measured according to McCormick (1993) and expressed relative to total homogenate protein which was measured using Bradford reagent (Bio-Rad, Richmond, CA, USA) and bovine serum albumin as a standard.

$J_{in}^{Na}$ was measured by adding 1.5 μCi (56 kBq) $^{22}Na$ to each experimental chamber and taking a 5 ml water sample at the respective time 0 (i.e. 0, 2, 6, 24 or 72 h after initiating exposure to hypercapnia) and 2 h following addition of isotope. Water samples were measured for radioactivity using a Beckman (Fullerton, USA) Coulter LS 6500 (Multi-purpose scintillation counter) for calculation of $J_{in}$ as indicated below. Water $Na^+$ (measured spectrophotometrically), $Cl^-$ (measured according to Zall et al., 1956), and total ammonia (measured using a Sigma diagnostic kit) were determined for calculation of net fluxes based upon the difference of the respective parameter over the 2 h incubation duration, taking into account the weight of individual fish.

### *Calculations*

Plasma [$HCO_3^-$] was calculated from the measured blood pH and $P_{CO_2}$ (Series II). In series I, plasma [$HCO_3^-$] was calculated from pH and total $CO_2$ concentration of plasma, using the Henderson–Hasselbalch equation. The $CO_2$ solubility coefficient and pK′ for plasma were taken from Boutilier et al. (1984). Intracellular [$HCO_3^-$] for heart, liver and muscle were calculated from the $pH_i$ of the respective tissue, and the $CO_2$ solubility coefficient and pK′ for plasma as described by (Heisler, 1982; Heisler et al., 1982).

$J_{in}^{Na}$ was calculated from the disappearance of isotope from the water and the average $Na^+$ concentration of the water during the flux period using the equation from Gonzalez and Dunson (1987):

$$J_{in} = \frac{(\ln Q_{out0} - \ln Q_{out1})Q_{out}}{(Mt)}, \quad (1)$$

where $Q_{out0}$ and $Q_{out1}$ are the total counts per minute (c.p.m.) in the flux chambers at the beginning and end of the flux period, respectively. $Q_{out}$ is the average amount of $Na^+$ in the flux bath during the flux period, $M$ is the mass of the fish in g, and $t$ is the time in h.

### *Statistics*

Differences among mean values within a given $P_{CO_2}$ treatment were determined using a one-way ANOVA, or a one-way repeated-measures ANOVA as appropriate, followed by a Tukey's *post-hoc* test. Data are presented as mean ± S.E.M., $N$=6, unless otherwise indicated.

## Results

### *Series I: Effect of hypercapnia on extracellular acid–base status*

Blood $P_{CO_2}$ remained elevated throughout the exposures to hypercapnia (Fig. 1A). When compared to normocapnia, extracellular pH was significantly reduced throughout all hypercapnic exposures. However, at a $P_{CO_2}$ of 14 mmHg, $pH_e$ increased significantly at 24 h compared to the initial and maximal reduction at 2 h, while $pH_e$ increased significantly at 96 h compared to 2 h during exposure to a $P_{CO_2}$ of 42 mmHg (Fig. 1B). Hypercapnia was associated with a progressive elevation of plasma $HCO_3^-$ that was more marked at the more severe exposure (Fig. 1C). When expressed in a pH–$HCO_3^-$

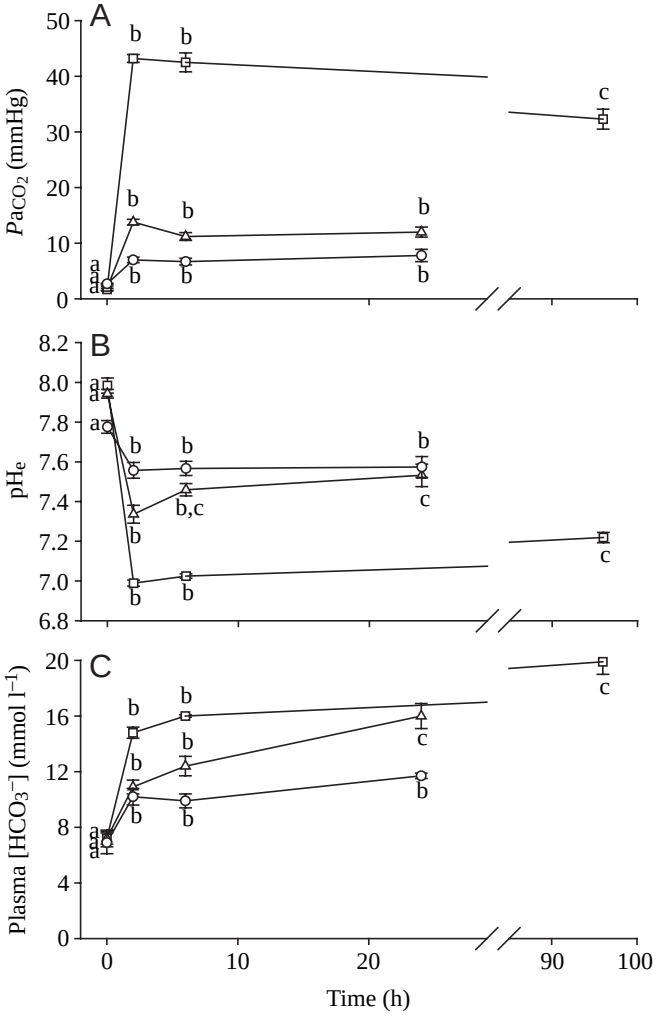


Fig. 1. The effect of exposure to a water $P_{CO_2}$ of 7 mmHg (circles), 14 mmHg (triangles) and 42 mmHg (squares) on (A) blood $P_{CO_2}$, (B) blood pH ($pH_e$) and (C) plasma $HCO_3^-$ in *L. pardalis*. Within each $P_{CO_2}$ treatment, lower case letters indicate values that differ significantly from each other.

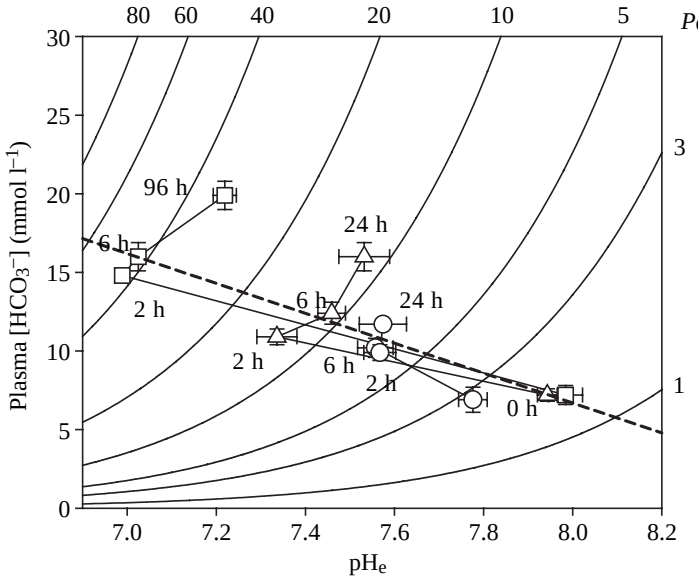


Fig. 2. pH–$HCO_3^-$ plot of blood from *L. pardalis* exposed to a $P_{CO_2}$ of 7 (circles), 14 (triangles) and 42 (squares) mmHg for up to 96 h. The broken line represents the *in vitro* buffer line for rainbow trout whole blood (Wood et al., 1982), and curved lines indicate $P_{CO_2}$ isopleths.

diagram (Fig. 2), and assuming that the whole blood non-bicarbonate buffer line of *L. pardalis* is similar to trout, it is apparent that half of the $HCO_3^-$ accumulation in plasma can be ascribed to non-bicarbonate buffering (Fig. 2). $pH_e$ did not recover at any level of hypercapnia. When expressed relative to the difference between $pH_e$ in normocapnia and that after 2 h, $pH_e$ only recovered by 8% following 24 h exposure to 7 mmHg, 32% following 24 h at14 mmHg, and 23% following 96 h exposure to 42 mmHg.

Hypercapnia did not affect arterial $P_{O_2}$ (pooled value=45.4±2.7mmHg), mean cell haemoglobin concentration (MCHC) (pooled value=3.9±0.1), plasma [$Na^+$] (pooled value=140.5±3.0 meq $l^{-1}$) or [$Cl^-$] (pooled value=114.6±1.1 meq $l^{-1}$). There was, however, a statistically significant reduction in Hct (from 40.4±1.6 at time 0, to 30.9±3.4 at time 24 h) which, most likely, relates to blood sampling. There were no significant changes in Hct in Series II.

### *Series II: Effect of hypercapnia on intracellular acid–base status*

When non-cannulated fish were exposed to hypercapnia, blood was withdrawn from the caudal vein following terminal anaesthesia to prevent struggling and associated acidosis. Nevertheless, $pH_e$ of normocapnic fish was lower than $pH_e$ of cannulated fish in Series I (Figs 1 and 3). Exposure to 14 mmHg significantly reduced $pH_e$ and red cell $pH_i$ at 6 and 24 h with no sign of pH compensation over 24h (Fig. 3). There were however, no significant changes in $pH_i$ of heart, liver or white muscle (Fig. 3). When exposed to 32 mmHg, *L. pardalis* also experienced a substantial, and largely uncompensated, reduction in $pH_e$ and red cell $pH_i$ that persisted for up to 72 h (Fig. 4). Again, however, there were no statistically significant reductions in $pH_i$ of heart, liver or white muscle during the 72 h of hypercapnia. Intracellular pH regulation of heart, liver and white muscle during the extracellular acidosis is evident when $pH_i$ is plotted against $pH_e$ for all time periods at both levels of hypercapnia (Fig. 5). Clearly, red cell pH decreases with lowered $pH_e$, while $pH_i$ of liver heart and skeletal muscle tend to increase.

As in series I, plasma $HCO_3^-$ increased during hypercapnia

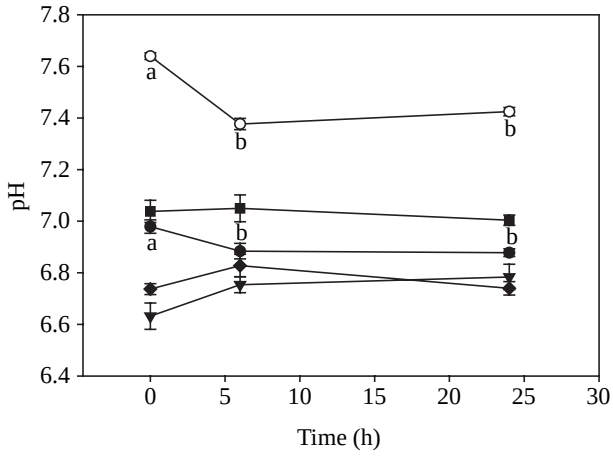


Fig. 3. The effect of exposure to a water $P_{CO_2}$ of 14 mmHg on whole blood pH (open circles) and intracellular pH of red blood cells (closed circles), heart (triangles), liver (diamonds) and muscle (squares) in *L. pardalis*. Letters that differ within a tissue differ significantly from one another. No significant differences were observed at any time in heart, liver and muscle.

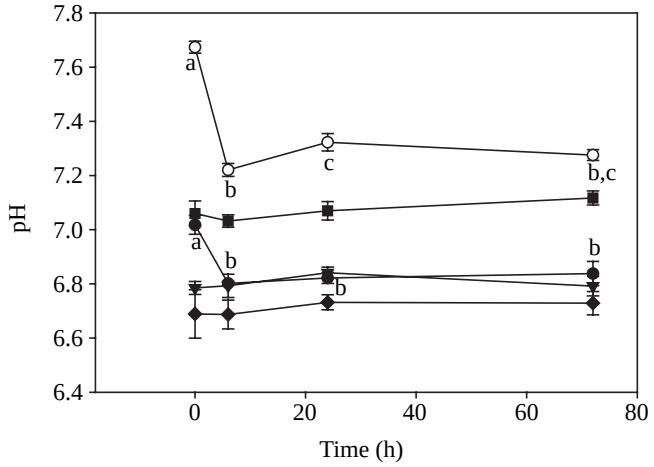


Fig. 4. The effect of exposure to a water $P_{CO_2}$ of 32 mmHg on whole blood pH (open circles) and intracellular pH of red blood cells (closed circles), heart (triangles), liver (diamonds) and muscle (squares) in *L. pardalis*. Letters that differ within a tissue differ significantly from one another. No significant differences were observed at any time in heart, liver and muscle.

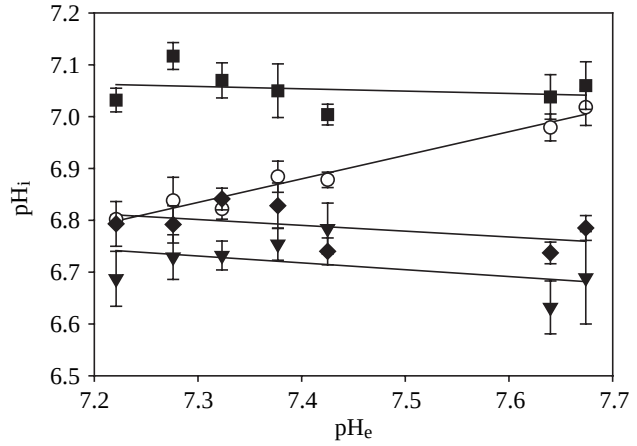


Fig. 5. The relationship between tissue intracellular pH ($pH_i$) and whole blood pH ($pH_e$) in *L. pardalis* exposed to a water $P_{CO_2}$ of 14 and 32 mmHg for up to 24 h. Circles represent red blood cells ($y$= 3.516+0.4546$x$, $r^2$=0.964), triangles represent heart ($y$=7.692–0.132$x$, $r^2$=0.211), diamonds represent liver ($y$=7.618–0.111$x$, $r^2$=0.247) and squares represent white muscle ($y$=7.388–0.045$x$, $r^2$=0.045).

(Table 1). There were no significant changes in plasma [$Na^+$], [$Cl^-$] or [$Ca^{2+}$]; however, there was a trend toward a reduction in plasma [$Cl^-$], consistent with the changes in plasma $HCO_3^-$. There was no significant effect of hypercapnia on haematocrit (pooled value=27.9±1.5), mixed-venous $P_{O_2}$ (pooled value=19.4±1.6 mmHg), plasma urea (pooled value= 513±56 μmol $l^{-1}$ nitrogen), plasma lactate (pooled value= 0.41±0.13 mmol $l^{-1}$), plasma glucose (pooled value= 3.0±0.2 mmol $l^{-1}$) or gill $Na^+$,$K^+$-ATPase activity (pooled value=0.82±0.32 mmol $l^{-1}$ $g^{-1}$ $h^{-1}$). There was a significant increase in plasma ammonia levels at both levels of hypercapnia (Table 1). Calculated intracellular [$HCO_3^-$] for heart, liver and kidney were significantly elevated at 6 h in both levels of hypercapnia. There were no significant differences after 6 h, indicating complete intracellular acid–base regulation at this time (Table 1).

While most of the intracellular $HCO_3^-$ accumulation during hypercapnia is due to non-bicarbonate buffering, additional $HCO_3^-$ accumulation must have occurred to compensate for the acidosis. Assuming a non-bicarbonate buffer value of 45 slykes for white muscle, as determined for the facultative air-breather, *Synbranchus marmoratus* (Heisler, 1982), it can be estimated that intracellular [$HCO_3^-$], in the absence of intracellular pH compensation, would have risen to approximately 5.5 mmol $l^{-1}$ at a $Pa_{CO_2}$ of 14 mmHg (A in Fig. 6). When compared to the calculated intracellular value of 6.8 mmol $l^{-1}$ (Table 1), 1.3 mmol $l^{-1}$ $HCO_3^-$ must have accumulated in the white muscle within 6 h to alleviate the acidosis. At a $Pa_{CO_2}$ of 32 mmHg, intracellular [$HCO_3^-$] would be expected to increase to approximately 9.2 mmol $l^{-1}$ in the absence of $pH_i$ compensation (B in Fig. 6). When subtracted from the calculated intracellular value of 13.0 mmol $l^{-1}$ (Table 1), it appears that 3.8 mmol $l^{-1}$ $HCO_3^-$ accumulated in white muscle to restore $pH_i$.

Table 1. *Series II plasma parameters and calculated intracellular [$HCO_3^-$] in heart, liver and white muscle in fish exposed to a water $P_{CO_2}$ of 14 or 32 mmHg for the indicated duration*

| | Plasma parameters | | | | | | Tissue [$HCO_3^-$] (mmol $l^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | $P_{CO_2}$ (mmHg) | Osmolarity (mOsm $l^{-1}$) | [$Na^+$] (μeq $l^{-1}$) | [$Cl^-$] (μeq $l^{-1}$) | [$Ca^{2+}$] (μeq $l^{-1}$) | $HCO_3^-$ (mmol $l^{-1}$) | [Ammonia] (μmol $l^{-1}$) | Heart | Liver | Muscle |
| 14 mmHg | | | | | | | | | | |
| 0 | 5.5±0.3[a] | 238.0±3.5 | 135.4±5.3 | 111.8±3.4 | 3.6±0.2 | 9.8±0.6[a] | 209.7±25.3[a] | 0.7±0.1[a] | 1.0±0.1[a] | 2.2±0.3[a] |
| 6 | 16.9±0.8[b] | 239.2±2.8 | 130.0±5.0 | 107.1±3.2 | 3.6±0.1 | 15.3±0.6[b] | 455.4±29.7[b] | 3.2±0.4[b] | 4.0±0.7[b] | 6.8±0.8[b] |
| 24 | 14.9±0.3[c] | 230.2±5.0 | 131.4±2.4 | 106.5±2.3 | 3.6±0.1 | 15.3±0.6[b] | 412.8±44.9[b] | 3.1±0.3[b] | 2.7±0.2[b] | 5.2±0.3[b] |
| 32 mmHg | | | | | | | | | | |
| 0 | 4.5±0.2[a] | 247.7±6.6 | 135.9±6.0 | 111.1±4.7 | 3.4±0.2 | 8.8±0.3[a] | 422.2±28.1[a,b] | 0.7±0.1[a] | 0.9±0.1[a] | 1.9±0.2[a] |
| 6 | 33.4±1.2[b] | 246.8±5.2 | 134.8±2.8 | 108.4±2.5 | 4.0±0.5 | 20.6±1.7[b] | 693.2±83.4[a,b] | 5.4±0.7[b] | 7.0±0.8[b] | 13.0±1.0[b] |
| 24 | 31.9±1.3[b] | 242.3±2.3 | 127.7±2.9 | 104.2±1.9 | 3.4±0.3 | 25.9±3.1[b] | 768.7±127.1[a,b] | 5.6±0.4[b] | 7.3±0.3[b] | 13.3±1.0[b] |
| 72 | 31.9±1.3[b] | 238.3±6.8 | 135.1±5.4 | 102.7±6.8 | 3.8±0.1 | 22.7±1.9[b] | 636.2±49.3[b] | 6.0±0.7[b] | 6.7±0.9[b] | 15.9±0.9[b] |

Different superscript letters indicate values significantly different from each other for each parameter within each $P_{CO_2}$ treatment.

*Series III: Effect of hypercapnia on whole animal ion fluxes*

Fish were exposed to hypercapnia simultaneously with those of Series II and thus experienced identical levels of hypercapnia. At a $P_{CO_2}$ of 14 mmHg, fish exhibited about a 70% reduction in $J_{in}^{Na}$ in conjunction with a large reduction in $J_{out}^{Na}$. As a result $J_{net}^{Na}$ did not change at 6 and 24 h (Fig. 7). There was a significant decrease in $J_{net}^{Cl}$, but no significant effect of hypercapnia exposure on total ammonia excretion (pooled value of 396±28 nmol $g^{-1}$ $h^{-1}$). Urea excretion rates prior to hypercapnia were low (11.5± 1.0 nmol $g^{-1}$ $h^{-1}$) representing a total of 8.0±0.9% of total nitrogenous waste excreted. Urea excretion was not measured during hypercapnia.

The effects of exposure to 32 mmHg were qualitatively similar with large reductions in both $J_{in}^{Na}$ and $J_{out}^{Na}$, resulting in no significant change in $J_{net}^{Na}$ over 72 h (Fig. 8). A significant reduction in $J_{net}^{Cl}$, relative to normocapnic control fish, was observed during exposure to hypercapnia. However, after 6 h, $J_{net}^{Cl}$ was near 0.

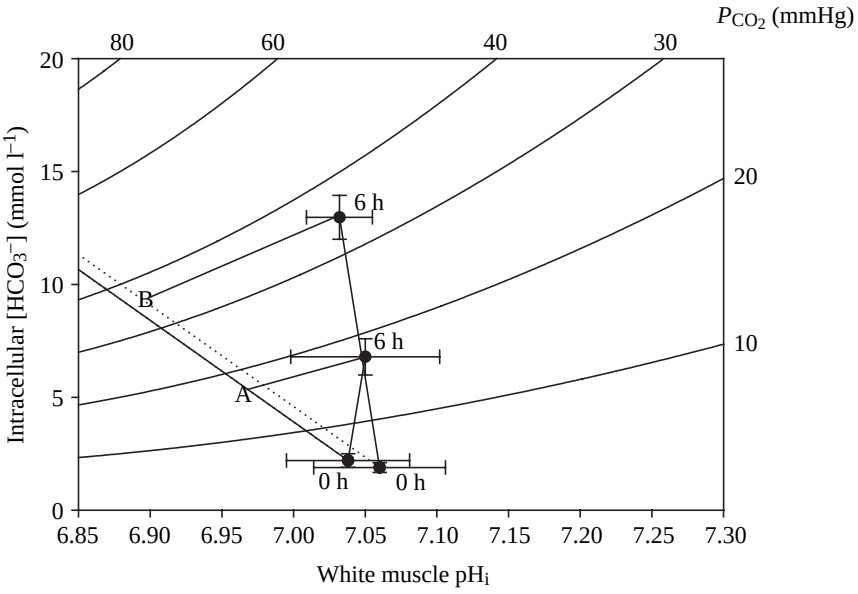


Fig. 6. pH–$HCO_3^-$ plot of white muscle intracellular fluid of *L. pardalis* exposed to a $P_{CO_2}$ of 14 and 32 mmHg for 6 h. The dotted line represents the *in vitro* buffer line for the white muscle of *Synbranchus marmoratus* (Heisler, 1982); curved lines indicate $P_{CO_2}$ isopleths. A and B indicate the predicted $HCO_3^-$ concentrations in white muscle in the absence of pH compensation at a water $P_{CO_2}$ of 14 and 42 mmHg, respectively.

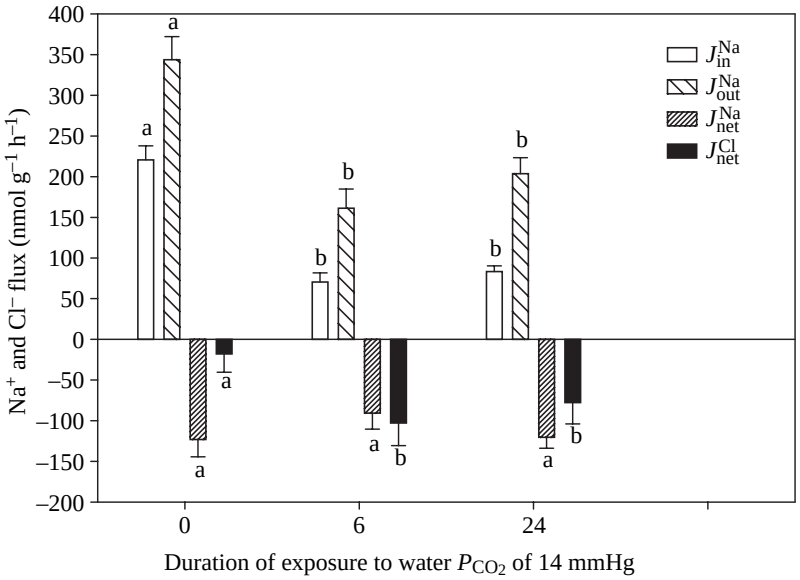


Fig. 7. Unidirectional $Na^+$ uptake ($J_{in}$) or loss ($J_{out}$) and net ($J_{net}$) $Na^+$ and $Cl^-$ flux during exposure to a water $P_{CO_2}$ of 14 mmHg for 24 h in *L. pardalis*. Letters that differ within a given parameter differ significantly from one another.

## Discussion

When *L. pardalis* were exposed to environmental hypercapnia, arterial pH was reduced in proportion to the rise in $Pa_{CO_2}$, with little subsequent accumulation of plasma $HCO_3^-$ and almost no compensation of $pH_e$ over the following 24–96 h. In spite of the pronounced extracellular acidosis (reaching a level as low as 6.99), $pH_i$ of the heart, liver and white muscle was tightly regulated within 6 h (the earliest time at which these parameters were measured). While most fish regulate $pH_i$ during exposure to environmental hypercapnia, the time course for this is often similar to that for $pH_e$ regulation (Heisler, 1984). The degree of extracellular acidosis tolerated by *L. pardalis*, and the ability to regulate $pH_i$ in the face of an extracellular acidosis, are among the greatest reported to date in a teleost fish.

*Extracellular acid–base regulation*

The magnitude of the respiratory acidosis tolerated by *L. pardalis* is among the greatest reported in the literature. Blood pH fell from 7.98 to 6.99 within 2 h of exposure to a water $P_{CO_2}$ of 42 mmHg. Eel (*Anguilla anguilla*) is also remarkably tolerant to hypercapnia, and blood pH fell from 7.92 to 7.16 as $P_{CO_2}$ increased from 3 to 45 mmHg over a 3 h period (McKenzie et al., 2002), resulting in no mortality with continued exposure to hypercapnia (McKenzie et al., 2003). Following 6 weeks exposure to a $P_{CO_2}$ of 45 mmHg, there was considerable $HCO_3^-$ accumulation (up to 72 mmol $l^{-1}$) associated with a large fall in plasma $Cl^-$ in the eel (McKenzie et al., 2003); however, restoration of $pH_e$ did not exceed 50%. White sturgeon *Acipenser transmontanus* also tolerate a

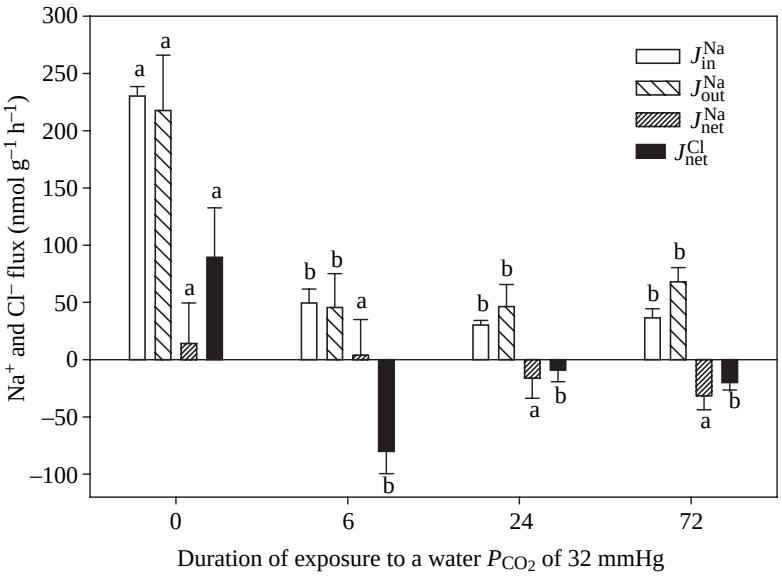


Fig. 8. Unidirectional $Na^+$ uptake ($J_{in}$) or loss ($J_{out}$) and net ($J_{net}$) $Na^+$ and $Cl^-$ flux during exposure to a water $P_{CO_2}$ of 32 mmHg for 72 h in *L. pardalis*. Letters that differ within a given parameter differ significantly from one another.

reduction in $pH_e$ from 7.7 to 7.15 when exposed to severe hypercapnia where arterial $P_{CO_2}$ reaches 30 mmHg (Crocker and Cech, 1998).

Most fish completely or partially restore $pH_e$, through net accumulation of plasma $HCO_3^-$ in exchange for $Cl^-$, within 10 to 72 h of being exposed to hypercapnia. Sturgeon, however, show a very blunted recovery of pH during hyepercapnia, where exposure to a $P_{CO_2}$ of 30 mmHg resulted in no $pH_e$ compensation at 24 h, and a mere 35% restoration of $pH_e$ following 72 h (Crocker and Cech, 1998). The water used in this study was relatively hard, which in many species facilitates acid–base regulation during hypercapnia (Heisler, 1984; Larsen and Jensen, 1997). The degree of pH compensation observed in *L. pardalis* during hypercapnia was similar to sturgeon. 24 h after exposure to 7 mmHg, $pH_e$ had only recovered by 8% and 96 h exposure to 42 mmHg merely led to a 23% recovery of $pH_e$. Hypercapnia was associated with a small reduction in $J_{net}^{Cl}$, indicating that branchial $Cl^-/HCO_3^-$ exchange was involved in the small rise of intra- and extracellular $HCO_3^-$. There was also a significant reduction of $J_{in}^{Na}$, which may be a direct effect of the low water pH (Wood, 1989). When *L. pardalis* were exposed to a $P_{CO_2}$ of 10 mmHg in the presence of a 60- to 70-fold elevation in water NaCl concentration (1.0 mmol $l^{-1}$ NaCl), there was no change in the rate or magnitude of acid–base recovery within 24 h (data not shown), indicating that the blunted extracellular response to hypercapnia may not be solely due to limitations associated with ionic composition of the water.

*L. pardalis* can tolerate a water $P_{CO_2}$ of 42 mmHg for weeks in water with low ionic levels, but it is not known whether they do compensate extracellular acid–base status over this duration. Interestingly, the ability of *L. pardalis* to tolerate extreme hypercapnia was size-dependent; in non-cannulated fish, all fish greater than 150 g survived, while all those weighing between 50–80 g died. Cannulation also resulted in higher mortality. Fish reduced spontaneous activity and remained stationary for days during exposure to severe hypercapnia. Although *L. pardalis* is a facultative air-breather, they did not attempt to air-breathe even when they had access to air (C.J.B., unpublished observation). It has been proposed that the exceptional tolerance to environmental hypercapnia observed in the eel is associated with the need to tolerate air exposure (Heisler, 1982; McKenzie et al., 2002). This may also be the case for *L. pardalis*, which can survive aerial exposure for days (A. L. Val, unpublished data).

A complete lack of extracellular pH regulation during the respiratory acidosis that develops upon transition to air-breathing was observed in the facultative air-breather, *Synbranchus marmoratus* (Heisler, 1982). When exposed to aquatic hypoxia for 4 days, *S. marmoratus* relied exclusively upon air-breathing which, in combination with the reduction of water flow across the gills, led to a fivefold increase of $Pa_{CO_2}$ and a reduction in $pH_e$ from about 8.15 to 7.5. Plasma $HCO_3^-$ did not increase to compensate for the acidosis, and there was very limited [$HCO_3^-$] uptake from the water. This lack of $pH_e$ compensation was concluded to result from the lack of gill–water contact and the associated impairment of branchial transfer of acid–base relevant ions (Heisler, 1982). It remains, however, to be established whether exposure to hypercapnic water, where gill–water interaction is maintained, would be associated with $pH_e$ compensation in *S. marmoratus*. *Liposarcus pardalis* is also a facultative air-breather and it is possible that its limited compensation of $pH_e$ during hypercapnia is associated with this trait rather than with the low ionic content of the water in which the fish lives. However, not all facultative air-breathers that experience a respiratory acidosis upon air-breathing exhibit a lack of pH compensation as seen in *S. marmoratus*. In another Amazonian catfish, *Hypostomus* sp., air-breathing was associated with a large reduction in gill ventilation (and presumably gill–water interaction) and blood $P_{CO_2}$ increased from 3 to 20 mmHg; however, $pH_e$ was fully compensated after 4–7 days due to a 15 mmol $l^{-1}$ rise of plasma [$HCO_3^-$], in spite of the low ionic content of the water (Wood et al., 1979).

### *Intracellular acid–base regulation*

Regulation of intracellular pH is important for enzyme function and metabolism and $pH_i$ is tightly regulated in most animals. Regulation of $pH_i$ is generally associated with partial to complete $pH_e$ compensation during an acid–base disturbance in most animals (Heisler, 1984). A reduction in $pH_i$ from 6.95 to 6.57 decreases glycolysis by 71% in mammalian cardiac muscle (see Somero and White, 1985). Severe impairment of aerobic metabolism is also likely with relatively small reductions of $pH_i$ (0.1–0.3 pH units), due to the pH

sensitivity of enzymes such as citrate synthase and pyruvate dehydrogenase (see Hazel et al., 1978). The effects of intra- and extracellular acidoses can be partially alleviated by a high tissue buffer value, achieved through an elevation in histidyl imidazole or phosphate groups (Somero and White, 1985); but $pH_i$ compensation requires active regulation.

Many teleost fishes regulate red cell pH during extracellular acidosis through release of catecholamines that stimulate red cell $Na^+/H^+$ exchange. This acts to safeguard oxygen transport, due to the presence of extremely pH-sensitive haemoglobins. This response protects blood oxygen binding that would have been reduced through the Root effect (see Nikinmaa, 1990). The armoured catfish do not exhibit a Root effect and do not posses adrenergic stimulation of red cell $Na^+/H^+$ exchange (Val et al., 1998). In contrast to the other tissues studied in *L. pardalis*, red blood cell pH decreased throughout the hypercapnic exposures. The ratio of the $\Delta pH_i/\Delta pH_e$ was 0.45, which is similar to that of other tissues in animals that experience a large extracellular acidosis over a 24–48 h period, and similar to that observed in red blood cells of rainbow trout *in vitro* in the absence of catecholamines (Heming et al., 1986). Turtle hearts, *in vitro*, exhibit a $\Delta pH_i/\Delta pH_e$ of approximately 0.55 (Jackson et al., 1991) and whole body $pH_i$ of the catfish *Ictalurus punctatus* is reduced with a $\Delta pH_i/\Delta pH_e$ of 0.60 during hypercapnia (Cameron, 1980), following which changes in $pH_e$ and $pH_i$ occurred roughly in parallel. In the skate exposed to a $P_{CO_2}$ of 7 mmHg, $\Delta pH_i/\Delta pH_e$ values for brain, heart and white muscle are approximately 0.39, 0.45 and 0.76, respectively, over a 24 h period, where recovery of $pH_i$ is tightly correlated with that of $pH_e$. During continued exposure to hypercapnia (days to weeks), $pH_i$ has been reported to be completely regulated in aquatic animals; however, this requires some degree of extracellular compensation, usually approaching 50% (Claiborne and Heisler, 1986; Pörtner et al., 1998; McKenzie et al., 2003).

The only two vertebrates reported to date that regulate $pH_i$ (of heart and white muscle) in the face of a large, uncompensated, extracellular acidosis are *S. marmoratus* (Heisler, 1982) and the salamander *Siren lacertina* (Heisler et al., 1982). In the salamander, an increase in water pH or bicarbonate infusion did not alter the acid–base strategy, indicating that the animals do not attempt to regulate $pH_e$, and preferentially regulate $pH_i$. In both *S. marmoratus* and salamanders, $pH_i$ of the heart and muscle was tightly regulated within 4 days, the earliest time measured. In the heart, liver and white muscle of *L. pardalis*, there was a trend towards an increase in $pH_i$ during the pronounced extracellular acidosis, which occurred as early as 6 h, indicating that active pH regulation must be involved. It is not known whether *S. marmoratus* or *S. lacertina* are capable of regulating $pH_i$ this rapidly.

The rapid, preferential regulation of $pH_i$ in the face of a largely uncompensated extracllular acidosis in *L. pardalis* is rare among vertebrates. It is not known whether this trait is associated with the ability to air-breathe and tolerate aerial exposure, with the low ionic content of the water, or with other environmental or evolutionary selective pressures. It is conceivable that this strategy could be selected in order to minimize problems associated with $Cl^-$ homeostasis in an aquatic environment such as the Amazon, which is dilute in ions (particularly $Cl^-$ and $HCO_3^-$), acidic, and prone to daily oscillations in hypercapnia. Complete $pH_e$ compensation during environmental hypercapnia is associated with large reductions in plasma $Cl^-$ levels that would have to be replaced rapidly when the fish moves from a hypercapnic to normocapnic environment. The ubiquity of this strategy among Amazonian fishes preferentially to regulate $pH_i$ and not regulate $pH_e$ is clearly worthy of further study.

### *Mechanisms of intracellular pH regulation*

Intracellular pH regulation is associated with a net $HCO_3^-$ uptake into the tissue, which is accomplished by active membrane transport of strong ions (Reeves, 1985). The $HCO_3^-$ accumulated may ultimately come from the water in association with gill $HCO_3^-/Cl^-$ or $Na^+/H^+$ exchange. The $J_{net}^{Cl}$ required to account for the intracellular $HCO_3^-$ accumulation during hypercapnia can be calculated, but is only reported for white muscle here. White muscle was the single largest tissue measured, and because white muscle $pH_i$ is higher than heart and liver, it is likely to represent a large proportion of the total intracellular $HCO_3^-$ accumulated by *L. pardalis* during hypercapnia. Assuming that white muscle comprises about 50% of body mass in *L. pardalis* (lower than other teleosts because of the dense bone of the skull), and that intracellular water represents about 85% of the white muscle mass, the active accumulation of 1.3 mmol $l^{-1}$ $HCO_3^-$ (see Results, Section II), requires that approximately 0.55 μmol $HCO_3^-$ $g^{-1}$ must have been accumulated within the white muscle 6 h after exposure to a water $P_{CO_2}$ of 14 mmHg. Assuming that this $HCO_3^-$ ultimately came from the water in exchange for $Cl^-$, then a $J_{net}^{Cl}$ of –92 nmol $g^{-1}$ $h^{-1}$ would be required to regulate white muscle $pH_i$, which is very similar to the measured $J_{net}^{Cl}$ value of –102 nmol $g^{-1}$ $h^{-1}$ (Fig. 7). Thus, based upon the fluxes measured in this study, and consistent with studies on other species (Heisler, 1984; Larsen and Jensen, 1997), branchial $HCO_3^-/Cl^-$ exchange is most likely to account for intracellular acid–base regulation under hypercapnia in *L. pardalis*.

Net $Cl^-/HCO_3^-$ exchange across the gills, however, does not appear to be sufficient to account for muscle $HCO_3^-$ accumulation at a higher $P_{CO_2}$. If 3.8 mmol $l^{-1}$ $HCO_3^-$ was actively accumulated in the white muscle at 32 mmHg (Table 1), and given the assumptions above, 1.6 μmol $HCO_3^-$ per gram of fish would have to be accumulated in the white muscle over the 6 h exposure duration, which would require a $J_{net}^{Cl}$ of –267 nmol $g^{-1}$ $h^{-1}$. This is far greater than the highest rate of –80 nmol $g^{-1}$ $h^{-1}$ measured at 6 h during this exposure, indicating that other pathways must be involved. Hypercapnia did not affect net $Na^+$ uptake or ammonia excretion and *L. pardalis* does not appear to excrete acid *via* the kidney (Randall et al., 1996). Thus, the remaining $HCO_3^-$ taken up by the muscle (and other tissues) may have been shuttled from the

extra to intracellular space. A reduction in plasma [$HCO_3^-$] below the blood buffer line was observed at 2 h, indicating that this may have occurred. A reduction in plasma [$HCO_3^-$] and an increase in plasma [$Cl^-$] were observed in *S. marmoratus* during initial exposure to hypercapnia, illustrating the preference of $pH_i$ over $pH_e$ regulation (Heisler, 1982). *Liposarcus pardalis* is endowed with a high bone mass, predominantly skull, and given the role of the shell and bone in compensating for an acidosis in turtles (Jackson, 1997; Jackson et al., 2000), bone demineralization may be playing a role in intracellular acid–base regulation in *L. pardalis*. Bone does not appear to be a route for acid–base compensation in the channel catfish, *Ictalurus punctatus*, exposed to hypercapnia (Cameron, 1985), and there were no statistically significant changes in plasma [$Ca^{2+}$] in *L. pardalis*; however, $Ca^{2+}$ efflux from the fish during hypercapnia was not measured. Whether or not bone demineralization is involved in acid–base regulation in *L. pardalis* remains to be investigated. Clearly $pH_i$ of the heart, liver, white muscle, and probably other tissues, is tightly regulated in the face of a large, predominantly uncompensated extracellular acidosis in *L. pardalis* during hypercapnia. Elucidating the precise mechanisms involved remains an exciting avenue for further studies.

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