Acid-base regulation in the air-breathing swamp eel (*Monopterus albus*) at different temperatures

Phan Vinh Thinh[1], Nguyen Thanh Phuong[1], Colin J. Brauner[2], Do Thi Thanh Huong[1], Andrew T. Wood[3], Garfield T. Kwan[4], Justin L. Conner[5], Mark Bayley[6] and Tobias Wang[6,7*]

[1] College of Aquaculture and Fisheries, Can Tho University, Can Tho City, Viet Nam

[2] Department of Zoology, University of British Columbia, 6270 University Blvd.,Vancouver, BC, V6T 1Z4 Canada

[3] CSIRO Agriculture and Food, Hobart, Australia

[4] Scripps Institution of Oceanography, University of California San Diego, USA

[5] University of Northern Texas, USA

[6] Zoophysiology, Department of Bioscience, Aarhus University, Aarhus, Denmark

[7]Aarhus Institute of Advanced Studies, 8000 Aarhus C, Denmark

*** Corresponding author**: Tobias Wang, +45 5137 7737, tobias.wang@bios.au.dk

Abstract

Vertebrates reduce arterial blood pH (pHa) when body temperature increases. In water-breathers this response occurs primarily by reducing plasma HCO_3^- levels with small changes in the partial pressure of CO_2 (PCO_2). In contrast, air-breathers mediate the decrease in pHa by increasing arterial PCO_2 ($PaCO_2$) at constant plasma HCO_3^- by reducing lung ventilation relative to metabolic CO_2 production. Much less is known in bimodal breathers that utilize both water and air. Here, we characterize the influence of temperature on arterial acid-base balance and intracellular pH (pHi) in the bimodal breathing swamp eel, *Monopterus albus*. This teleost uses the buccopharyngeal cavity for gas exchange and has very reduced gills. When exposed to ecologically relevant temperatures (20, 25, 30 and 35ºC) for 24 and 48h, pHa decreased by -0.025 pH units/°C (U/°C) in association with an increased $PaCO_2$, but without changes in plasma [HCO_3^-]. Intracellular pH (pHi) was also reduced with increased temperature. The slope of pHi of liver and muscle was -0.014 and -0.019 U/ºC, while the heart muscle showed a smaller reduction (-0.008U/ºC). When exposed to hypercapnia (7 or 14 mmHg) at either 25 or 35ºC, *Monopterus albus* elevated plasma [HCO_3^-] and therefore seemed to defend the new pHa set-point, demonstrating an adjusted control of acid-base balance with temperature. Overall, the effects of temperature on acid-base balance in *Monopterus albus* resemble air-breathing amniotes, and we discuss the possibility that this pattern of acid-base balance results from a progressive transition in CO_2 excretion from water to air as temperature rises.

Keywords: acid-base regulation, blood gases, temperature, intracellular pH, *Monopterus albus*

Summary statement: The air-breathing fish Monopterus albus reduces blood and tissue pH by increasing PCO_2 with elevated temperature in a manner that resemble tetrapods

Introduction

All animals, with a few notable exceptions, decrease the pH of their bodily fluids as temperature increases (*e.g.* Truchot, 1987; Ultch and Jackson, 1996; Stinner and Hartzler, 2000; Burton, 2002; Wang and Jackson, 2016). In aquatic ectothermic vertebrates, the reduction in blood pH is primarily associated with a reduction in the plasma HCO_3^- concentration ($[HCO_3^-]$) at constant partial pressure of CO_2 (PCO_2), whilst air-breathing vertebrates reduce blood pH by increasing PCO_2 through a reduction in ventilation relative to metabolic CO_2 production (Randall and Cameron, 1973; Larry, 1979 Austin et al., 1927; Smatresk and Cameron 1982; Cameron and Kormanik 1982; Boutilier et al. 1987; Amin-Naves et al. 2004). This marked difference in strategy probably stems from water-breathers being obliged to maintain high ventilation rates due to the low solubility of oxygen in water, whilst air-breathers are endowed with the luxury of modulating acid-status through ventilatory adjustments (Rahn, 1966). As an obvious benefit, the respiratory modulation of acid-base status obviates the need for transepithelial ion exchange and the associated osmotic disturbances that ensue (Austin et al 1927; Burton, 2002).

While differences between water and air-breathers remains a cherished topic among comparative physiologists, relatively little is known about the influence of temperature on acid-base status in bimodal breathers (e.g. air-breathing fishes that utilize both air and water for gas exchange). The transition from water to air-breathing is associated with an elevation of blood CO_2 levels due to the reduced ventilation in air relative to water (Rahn, 1966). Consequently, many air-breathing fish have much higher arterial PCO_2 ($PaCO_2$) levels than water-breathing fish (*e.g.* Cameron and Wood, 1978; Shartau and Brauner, 2014). This has implications for the influence of temperature on acid-base status because an increase in temperature is expected to increase the reliance on air-breathing as metabolism rises ("the Q_{10} effect"). Therefore, an increase in temperature is likely to induce a passive rise in $PaCO_2$ in bimodal breathers as the partitioning of gas exchange shifts from water to air. Indeed, two studies on the facultative air-breathing freshwater gar (*Lepisosteus oseus* and *Lepisosteus oculatus*) have demonstrated a clear and reversible elevation of $PaCO_2$ with an increase in temperature (Rahn et al., 1971; Smatresk and Cameron, 1982). Further, when forced to employ air-breathing through exposure to hypoxic water, the facultative air-breather *Pangasianodon hypophthalmus* also exhibits an increase $PaCO_2$ with an increase in temperature (Damsgaard et al., 2018).

The purpose of the present study was to extend our understanding of the influence of temperature on acid-base regulation in an air-breathing teleost, *Monopterus albus* (Asian swamp eel, Zuiew 1793). *Monopterus albus* is an obligate air-breathing teleost that thrives in muddy ponds, swamps, and other stagnant freshwater bodies in tropical South-East Asia. The metabolic rate of *Monopterus albus* conforms to a normal Q_{10} of around 2 (Lefevre et al., 2016). The gills of *Monopterus albus* are greatly reduced and have a low capacity for gas exchange. Instead, oxygen uptake occurs primarily over the highly vascularized epithelium of the buccopharyngeal cavity and esophagus, and facilitated by a very high blood oxygen affinity (Shih, 1940; Rainboth, 1996; Iversen et al., 2013; Damsgaard et al., 2014). Current climate models have suggested that temperature in the Mekong area might increase from 27^{o}C to as much as 33^{o}C within the coming century (MRC, 2009). As an air-breather with gills that possess a modest capacity for gas exchange, *Monopterus albus* has a rather high $PaCO_2$ (10-20 mmHg; Damsgaard et al., 2014) and have been reported to tolerate a broad thermal range (8-40^{o}C; Shafland et al., 2009; Lefevre et al., 2016). However, the effects of temperature on intracellular pH (pHi) changes are not known.

Our study had three specific aims. Firstly, we wished to establish how arterial acid-base balance and plasma ion concentrations were affected by temperatures within the range of 20 to 35^{o}C. These measurements were performed on chronically cannulated fish exposed to a progressive rise in temperature. In addition, although most proteins and their biological functions reside within the cells, much less is known about the influence of temperature on intracellular acid-base status. A second aim of our study was therefore to measure intracellular pH in separate groups of fish kept at four temperatures (20, 25, 30 and 35^{o}C). Whilst these previous two objectives describe the influence of temperature, our third aim was to establish whether the reduction in arterial pH with temperature is indeed regulated. To address this question, we exposed *Monopterus* to two levels of hypercapnia (7 and 14 mmHg in water and air) at both 25 and 35°C to investigate whether they defend pHa at a given temperature by metabolic compensation in response to the induced mild respiratory acidosis.

Materials and methods

2.1 Experimental animals

Asian swamp eels of both sexes and a mean body mass of 342 ± 9 g) (*Monopterus albus*) were purchased from a commercial farm in the Mekong Delta in southern Vietnam and kept at University of Can Tho for a 3-6 weeks before the experiments were performed. During this period, the eels were kept in aerated 300 L tanks maintained at 27°C and provided with stiff plastic tape as shelter. The experiments were conducted in accordance with rules and regulations of animal experiments in the European Union.

2.2 Surgery and catheterization of the dorsal aorta

The swamp eels were anaesthetized by submergence in a benzocaine solution (225 mg/L) for 20-30 min until they stopped spontaneous movement, and were then transferred to an operating table in a supine position. A 2-4 cm incision into the abdominal cavity provided access to the coeliac artery for cannulation with polyethylene tubing (PE50) containing heparinized saline (50 UI/1 mL saline). The incision was closed with stitches and the catheter was secured to the skin. The surgery lasted <20 min and eels were allowed to recover for 24 h individually in small tanks with aerated water at room temperature.

2.3 Experimental design

Series 1: Effects of temperature on arterial acid-base status

We measured arterial acid-base status and plasma ion concentrations in six eels as temperature was increased progressively from 20 to 25, 30 and 35°C. Each temperature was maintained for 48h where upon fish were sampled, and temperature gradually increased to the next target value over the subsequent 2-3 hours. Sampling consisted of drawing 1 ml of arterial blood from the dorsal aorta at 24h and 48h for each temperature level, which was analyzed immediately for pHa, $PaCO_2$, total CO_2 concentration of the plasma, Hb and Hct, while plasma samples were frozen for subsequent measurements of plasma ion concentrations and osmolality.

Series 2: Effects of temperature on intracellular pH

We measured pHi of liver, heart and muscle at each of the four temperatures (20, 25, 30 and 35°C). For each temperature, we performed measurements on six individuals that had been maintained at the relevant temperature for 24h. None of these fish were instrumented with catheters and had therefore not been anaesthetized prior to sampling. The eels were killed quickly by a sharp blow to the head, allowing rapid tissue removal (2-4 min) through an incision. All tissue samples were wrapped in aluminum foil before immersion in liquid N_2 after which they were held at -80°C until measurements were made.

Series 3: Effects of temperature on the metabolic compensation of arterial pH

After recovering from surgery, eels were maintained at either 25 or 35°C in aerated normoxic water. After drawing a 1 ml arterial blood sample (control), the eels were then exposed to either normocapnia, or hypercapnia (in both water and air simultaneously) of 7 or 14 mmHg CO_2 for 72h (six fish in each group). During these exposures, a 1 ml arterial blood sample was taken at 24, 48 and 72h. At both temperatures, we included an untreated control group that was maintained normocapnic in both air and water for the duration of the experiment. All blood samples were then analyzed for arterial acid-base status and plasma ion concentrations.

Analytical methods

pHa and $PaCO_2$ were measured with CG3+ cartridges on the iSTAT handheld blood gas analyser where values were temperature compensated to the fish temperature, using the equation from the iSTAT manual (i-STAT Corporation, Princeton, USA) (Harter et al., 2014; Damgaard et al., 2015). The total CO_2 concentration in the plasma was measured according to Cameron (1971), allowing for bicarbonate concentration in the plasma ($[HCO_3]_{pl}$) to be calculated by subtraction of dissolved CO_2 ($PaCO_2$ x α_{CO2}), using an appropriate α_{CO2} (Boutilier e*t al.*, 1985). Haemoglobin concentration ([Hb]) was measured spectrophotometrically at 540 nm after conversion to cyanmethaemoglobin using Drabkin's reagent, whilst haematocrit (Hct) was determined as the fraction of packed red cells following centrifugation at 12,000 rpm for 3 min. Plasma chloride concentration ($[Cl^-]$) was determined by titration using a Sherwood chloride analyzer (model 926S MK II), while sodium and potassium concentrations were measured by flame photometry (Sherwood Model 420, Sherwood Scientific Ltd., Cambridge, UK). Total

osmolality was measured on a Fiske Model 210 Micro Osmometer (Advanced Instruments™ Fiske™ 210 Micro-Sample Osmometer, USA).

To measure pHi, heart, liver, and muscle samples were ground to a fine powder under liquid N_2 (Portner et al., 1990; Brauner et al., 2004; Baker et al., 2009ab). Approximately 0.1 g of the powder was placed in a 1.5 ml Eppendorf tube containing 0.8 ml of metabolic inhibitor cocktail (150 mmol l^{-1} potassium fluoride (KF) and 6mmol l^{-1} nitrilotriacetic sodium; Na_2NTA). The solution was immediately vortexed for 30s and centrifuged at 3000 g in 45s. Then the pH of the supernatant (0.2 ml) was measured with a Mettler Toledo pH electrode. In all cases, the pH electrode was calibrated and maintained at the same temperature as the fish, and the supernatant was measured at this temperature.

2.4 Statistical analysis

A two-way ANOVA for repeated measures (a multivariate analysis of covariance) was used to test for overall effect of temperature and hypercapnia on blood parameters followed by DUNCAN's post hoc test to identify difference amongst means. To evaluate the isolated effects of temperature, a one-way ANOVA was used. Data are presented as mean ± standard error of the mean (mean ± S.E.M.). A probability (p) value of less than 0.05 was considered significant. All statistical analyses were performed using PASW Statistic 18.

Results

Series 1: Effects of temperature on arterial acid-base status

Arterial acid-base status in response to the progressive rise in temperature from 20 to 35°C is shown in Figure 1, along with plasma ion concentrations and osmolality in Table 1. The rise in temperature caused a virtually linear reduction in pHa with a slope of 0.025±0.001 and 0.028±0.001 pH units$^{o}C^{-1}$ after 24 and 48h at each temperature, respectively. These slopes did not differ significantly. The $PaCO_2$ rose progressively from 10.1±1.0 at 20°C to 19.1±1.0 at 35°C (p<0.05). Plasma [HCO_3^-] and ion concentrations were unaffected by temperature (Table 1). The concentration of haemoglobin and hematocrit were not affected by temperature (Table 2).

Series 2: Effects of temperature on intracellular pH

Increased temperature also led to a reduction in pHi (Fig. 2). In the cardiac tissue, pHi decreased from 7.45±0.04 (20°C) to 7.36 ± 0.04 (30°C) (p>0.05) and a stronger and significant fall to 7.32 ± 0.02 at 35°C (p<0.05). Over the same temperature range, liver tissue pHi decreased rapidly

from 7.29±0.03 (20°C) to 7.08±0.03 (35°C) (p<0.05). The skeletal muscle showed the lowest pHi values and fell significantly from 7.29±0.02 to 7.00±0.04 (p<0.05) over the same temperature range.

Series 3: Effects of temperature on the metabolic compensation of arterial pH

The regulation of arterial acid-base status during the two levels of hypercapnia (7 and 14 mmHg in water and air) at 25 and 35°C are shown in Figure 3 in comparison to the normocapnic controls. At 25°C, both levels of hypercapnia (7 and 14 mmHg) led to an elevation of $PaCO_2$, but pHa remained unaffected at 72h due to an elevation of HCO_3^- in the plasma. The effects of hypercapnia were less pronounced at 35ºC where $PaCO_2$ was higher and pHa lower than at 25ºC. Nevertheless, in response to 14 mmHg CO_2, there was still a significant rise in plasma [HCO_3^-] whilst pHa remained unaffected. Thus, while exposure to increased PCO_2 had little effect on pHa (p=0.944), increased temperature caused a significant reduction in pHa (p=0.00001). Further, there was a significant effect of the combination of temperature and PCO_2 (p=0.012). Plasma ion concentrations and osmolality remained unaffected by hypercapnia at either temperature (Table 3). Additionally, [Hb] and Hct did not change during hypercapnia (Table 4).

Discussion

Monopterus albus exhibited the archetypical reduction in plasma pH and a reduction in pHi of three major tissue-types with an increase in temperature. The magnitude of the pHa reduction was larger than reported for most vertebrates, but $\Delta pH/\Delta T$ in *Monopterus albus* is not exceptional (Malan et al. 1976, Heisler et al., 1976; Moalli et al., 1981; Cameron and Kormanil, 1982; Walsh and Moon, 1982; Boutilier et al. 1987; Amin-Naves et al. 2004; Fobian et al. 2014). The responses to temperature were relatively fast and appeared complete within 24h as there were no changes from 24 to 48h after the changes in temperature, but we cannot the short term effects (hours) upon temperature changes.

The pattern of acid-base regulation in *Monopterus albus* differs markedly from the typical water-breathing teleost pattern because the reduction in pH was mediated entirely by an elevation of $PaCO_2$ and plasma HCO_3^- remained unaffected. The influence of temperature on acid-base balance in *Monopterus albus* therefore resembles air-breathing vertebrates where the fall in pHa with increased temperature is achieved through a reduction in ventilation relative to CO_2

production, which alleviates the need for trans-epithelial ion exchange. Consistent with this pattern, plasma ion concentrations remained unaffected by temperature in *Monopterus albus*. This is the first clear report of this pattern in a teleost.

The pronounced rise in $PaCO_2$ of *Monopterus* with elevated temperature is larger than reported for other teleosts, but the overall response appears to reflect a general trend amongst the few air-breathing fishes that have been studied to date. In the lobe-finned South American lungfish (*Lepidosiren paradoxa*), $PaCO_2$ increases with temperature due to increased reliance on pulmonary gas exchange as metabolic CO_2 production rises (Amin-Naves *et al*., 2004). Similarly, in the non-teleost ray finned air-breathing garfish (*Lepisosteus osseus*), $PaCO_2$ is elevated during the higher summer temperatures compared to the colder winter months (Rahn et al., 1971). Within the teleost taxa, the facultative air-breathing striped catfish *Pangasianodon hypophthalmus* also elevates $PaCO_2$ with temperature when forced to air-breathe in hypoxic water (Damsgaard *et al*. 2018). However, in this case the elevation in $PaCO_2$ with elevated temperature is associated with a significant elevation of plasma [HCO_3^-], and Damsgaard *et al*. (2018) argued that the transfer of gas exchange from water to air is attended by a passive increase in $PaCO_2$, and the pH drop is fine-tuned by elevations in HCO_3^- through branchial ion exchange.

In *Monopterus albus*, the acid-base changes in response to temperature resemble the classic pattern of tetrapods and lungfishes where central chemoreception for CO_2 (and probably pH) in the cerebrospinal fluid plays a major role in reducing pulmonary ventilation relative to metabolic CO_2 production to elevate $PaCO_2$ as temperature rises (e.g. Hitzig and Jackson, 1978; Jackson, 1989; Branco et al., 1993; Amin-Naves et al., 2004). However, as a teleost fish, it seems unlikely that *Monopterus albus* is in possession of central CO_2 chemoreception (Milsom, 2010). Nevertheless, *Monopterus albus* exhibits a vigorous ventilatory response to hypercapnia (Mikkel Thomsen, Mark Bayley and Tobias Wang, unpublished observation), and the putative role of peripheral chemoreceptors should be considered in future studies on the influence of temperature on the ventilatory regulation of acid-base balance in air-breathing fishes.

A simple explanation for the rise in $PaCO_2$ and the associated reduction in pH in *Monopterus albus*, it is likely that the rise in metabolic CO_2 production with increased temperature imposes a transition from aquatic to aerial CO_2 excretion. In this scenario, the rise in $PaCO_2$ can be seen as a simple passive consequence of the increased reliance on air-breathing with increased

temperature (Lefevre et al., 2014) and the associated retention of CO_2 (Rahn, 1966). Similar responses are characteristic of amphibians where gas exchange is partitioned between the lungs and skin (Wang et al., 1998; Boutilier and Heisler, 1987), as well as in the lungless salamanders where the reduction in pHa with increased temperature is due to an elevation of $PaCO_2$ because cutanbeous conductance for CO2 changes very little with temperature (Moalli et al., 1981).

Following this argument, it was pertinent to investigate whether the temperature-induced change in pHa constitutes a new regulated set-point. To address this question, we exposed *Monopterus albus* to mild levels of hypercapnia at 20 and 30^{o}C. The results clearly show that *Monopterus albus* responds to the induced respiratory acidosis with an increase in plasma HCO_3^- concentration and thus regulates pHa to a temperature specific value. There remains therefore little doubt that temperature changes the pHa set point in a tightly regulated manner in this species. Further, this experiment also demonstrates that this species possesses the necessary capacity for compensation of the extracellular respiratory acidosis. This is a common trait amongst water-breathing fishes, but has been found lacking in some air-breathing fishes (Shartau and Brauner, 2014) though not in others (Damsgaard *et al*., 2015; Gam *et al.*, 2017).

The observation that the reduction in pHa is indeed regulated, however, provides little novel insight into the regulated variable. The magnitude of ΔpHa/ΔT in *Monopterus* is consistent with the notion of maintaining constant protein (mainly imidazole) ionization (e.g. Reeves, 1972), but this obviously does not provide conclusive evidence for protein ionization being the regulated variable. Nevertheless, it is striking that plasma HCO_3^- remained constant across temperature, which in combination with the lack of change in other extracellular ion concentrations, is consistent with the idea of constancy of protein ionization (Stewart, 1978). The biological significance of the reduction in pH with increased temperature is obviously of particular importance for the intracellular compartments where most protein function occurs (Malan et al., 1976; Reeves, 1977). Our measurements of pHi from muscle, liver and heart indicate that the increase in temperature affected intracellular acid-base status, but the ΔpHi/Δt were lower than for blood pH, with the heart being the least affected. These data contrast with other studies where the ΔpH/Δt of the heart was greater than other organs (muscle, liver and brain) (Table 5). However, Cameron and Kormanik (1982) reported ΔpHi/Δt to be lower in heart tissue compared to blood in *Ictalurus punctatus*. Heisler et al. (1976) also reported differences amongst organs in dogfish and carp (Heisler, 1980) and the lower ΔpHi/Δt in the cardiac tissue of *Monopterus* be

may be associated with its very high levels of myoglobin in the ventricle (Damsgaard et al., 2014).

In conclusion, we demonstrate that *Monopterus* reduces both pHa and pHi as temperature increases. The reduction in pH clearly seems to reflect a change in the regulated set-point. In contrast to water-breathing fishes, but in accordance with an emerging pattern amongst air-breathing fishes, the lowering of pH is accomplished by a rise in $PaCO_2$ that may be a passive consequence of increased reliance on the air-breathing organ for CO_2 excretion as increased temperature stimulates metabolic CO_2 production. It remains to be investigated, however, whether CO_2/pH sensitive chemoreceptors also play a role in this response; clearly an area for further research.

Competing interests

No competing interests declared.

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References

Aickin, C. C. and Thomas, R. C. (1977). Micro-electrode measurement of the intracellular pH and buffering power of mouse soleus muscle fibers. *J. Physiol.* **267**,791–810.

Amin-Naves J., Giusti, H. and Glass, M. L. (2004). Effects of acute temperature changes on aerial and aquatic gas exchange, pulmonary ventilation and blood gas status in the South American lungfish, *Lepidosiren paradoxa*. *Comp. Biochem. Physiol.* **138A**, 133–139.

Austin J. H., Sunderman F. W. and Camack J. G. (1927) Studies in serum electrolytes II. The electrolyte composition and the pH of serum of a poikilothermous animal at different temperatures. *J. Biol. Chem.* **72**, 677–685.

Baker, D. W., Matey, V., Huynh, K. T., Wilson, J. M., Morgan, J. D. and Brauner, C. J. (2009a). Complete intracellular pH protection during extracellular pH depression is associated with hypercapnia tolerance in white sturgeon, *Acipenser transmontanus*. *Am. J. Physiol.* **296**, R1868–R1880.

Baker, D. W., May, C. and Brauner, C. J. (2009b). A validation of intracellular pH measurements in fish exposed to hypercapnia: the effect of duration of tissue storage and efficacy of the metabolic inhibitor tissue homogenate method. *J. Fish Biol.* **75**, 268–275.

Boutilier, R. G., Iwama, G. K., Heming, T. A. and Randall, D. J. (1985). The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15 C. *Respir. Physiol*. **61**, 237-254.

Boutilier, R.G., Glass M. L. and Heisler, N. (1987). Blood gases and extracellular/intracellular acid-base status as a function of temperature in the anuran amphibians *Xenopus laevis* and *Bufo marinus*. *J. Exp Biol.* **130**, 13-25.

Branco, L. G. S., Glass, M. L., Wang, T. and Hoffmann, A. (1993). Temperature and central chemoreceptor drive to ventilation in toad (*Bufo paracnemis*). *Respir. Physiol*. **93**, 337-346.

Brauner, C. J., Wang, T., Wang, Y., Richards, J. G., Gonzalez, R. J., Bernier, N. J., Xi, W., Patrick, M. and Val, A. L. (2004). Limited extracellular but complete intracellular acid-base regulation during short-term environmental hypercapnia in the armoured catfish, *Liposarcus pardalis*. *J. Exp. Biol.* **207**, 3381–3390.

Burton, R. F. (2002). Temperature and acid–base balance in ectothermic vertebrates: the imidazole alphastat hypotheses and beyond. *J. Exp. Biol.* **205**, 3587-3600.

Cameron J. N. and Kormanik G. A., (1982) Intracellular and extracellular acid-base status as a function of temperature in the freshwater channel catfish, *Ictalurus punctatus*. *J. Exp. Biol.* **99**,127–142.

Cameron, J. N., (1971). Rapid method for determination of total carbon dioxide in small blood samples. *J. Appl. Physiol*. **31**, 632-634.

Cameron, J. N. and Wood, C. M., (1978). Renal function and acid-base regulation in two Amazonian erythrinid fished: (*Hoplias malabaricus*), a facultative air breather. *Can. J. Zool.* **65**, 917-930

Damsgaard, C., Findorf, I., Helbo S., Kocagoz, Y., Buchanan, R., Huong, D. T. T., Weber, R. E., Fago A., Bayley, M. and Wang, T. (2014). Very high blood oxygen in the air-breathing swamp eel *Monopterus albus*. *Comp. Biochem. Physiol.* **178**, 102-108.

Damsgaard, C., Gam. L. T. H., Tuong. D. D., Thinh. P. V. , Huong. D. T. T, Wang. T. and Bayley. M. (2015). High capacity for extracellular acid-base regulation in the air-breathing fish P*angasianodon hypophthalmus*. *J. Exp. Biol.* **218**, 1290-1294

Damsgaard, C., Thomsen M. T., Bayley, M. and Wang, T. (2018). Air-breathing changes the pattern for temperature-induced pH regulation in a bimodal breathing teleost. *J Comp Physiol* (in press).

Ellis, D., and MacLeod, K. T. (1985). Sodium-dependent control of intracellular pH in Purkinje fibers of sheep heart. *J. Physiol.* **359**, 81–105

Fobian, D., Overgaard, J. and Wang, T. (2014). Oxygen transport is not compromised at high temperature in pythons. *J. Exp. Biol.* **217**, 3958-3961.

Gam, L.T.H., Jensen, F.B., Damsgaard, C., Phuong, N.T. and Bayley, M. (2017). Extreme nitrite tolerance in the clown knifefish *Chitala ornata* is linked to up-regulation of methaemoglobin reductase activity. *Aquat. Toxicol.* **187**, 9-17

Glass, M. L., Ishimatsu, A. and Johansen, K. (1986). Responses of aerial ventilation to hypoxia and hypercapnia in *Channa argus*, an air-breathing fish. *J. Comp. Physiol.* **156**, 425-430

Graham, J. B. (1997) Air-breathing fishes: evolution, diversity, and adaptation. Acedemic press, San Diego, CA, USA

Heisler, J. N., Weitz, H. and Weitz, A. M. (1976). Extra and intracellular pH with changes of temperature in the dogfish *Scryliorhinus stellaris*. *Respir. Physiol.* **26**, 249-264.

Heisler, N. (1980) Regulation of the acid-base status in fishes, in *Environmental Physiology of Fishes* (Ali, M.A., Ed.), pp. 123-162, Plenum, New York.

Heisler, N. (1982). Intracellular and extracellular acid-base regulation in the tropical fresh-water teleost fish *Synbranchus marmoratus* in response to the transition from water breathing to air breathing. *J. Exp. Biol.* **99**, 9-28.

Hitzig, B. M. and Jackson, D. C. (1978). Central chemical control of ventilation in the unanaesthetized turtle. *Am. J. Physiol*. **235**, R257-R264.

Howell, B. J., Baumgardner, W., Bondi, K. and Rahn, H. (1970). Acid-base balance in cold-blooded vertebrate as a function of temperature. *Am. J. Physiol.* **218**, 600-606.

Iversen, N. K., Lauridsen, H., Huong, D. T. T., Cong, N. V., Gesser, H., Buchanan, R., Bayley, M., Pedersen, M. and Wang, T. (2013). Cardiovascular anatomy and cardiac function in the air-breathing swamp eel (*Monopterus albus*). *Comp. Biochem. Physiol.* **164A**, 171-180.

Jackson, D. C. (1989). Control of breathing. Effects of temperature. In: *Comparative Pulmonary Physiology*, Vol. 39, edited by S.C. Wood. New York, Basel: Marcel Dekker, pp. 621-641.

Larry, I. C. (1979) Responses to rapid temperature change in Vertebrate Ectotherms. *Integr. Comp. Biol.* **19**, 225-237

Lefevre, S., Findorf, I., Bayley, M., Huong, D. T. T. and Wang, T. (2016). Increased temperature tolerance of the air-breathing Asian swamp eel *Monopterus albus* after high-temperature acclimation is not explained by improved cardio-respiratory performance. *J. Fish. Biol.*, **88**, 418-432

Lomholt, J. P. (1974). Control of breathing in *Amphipnous cuchia*, an amphibious fish. *Respir Physiol* **21**, 325-340.

Lomholt, J. P. and Johansen K. (1976). Gas exchange in the amphibious fish, *Amphipnous cuchia*. *J. Comp. Physiol. B* **197**, 141-157.

Malan, A., Wilson, T. L. and Reeves, R. B. (1976). Intracellular pH in cold-blooded vertebrates as a function of body temperature. *Respir. Physiol.* **17**, 45-61

Malte C. L., Jakobsen S. L. and Wang T. (2014). A critical evaluation of automated blood gas measurements in comparative respiratory physiology. *Comp. Biochem. Physiol.* **178**, 7–17.

Milsom, W. K. (2010). The phylogeny of central chemoreception. *Respir. Physiol.* **173**, 195-200.

MRC (2009). Adaptation to climate change in the countries of the Lower Mekong Basin: regional synthesis report. *MRC Technical Paper No. 24*. Vientiane: Mekong River Commission.

Moalli, R., Meyers, R. S., Ultsch, G. R. andJackson D. C., (1981). Acid-base balance and temperature in a predominantly skin-breathing salamander, *Crytobranchus alleganiensis*. *Respir. Physiol.* **43**, 1-11.

Pörtner, H. O., Boutilier, R. G., Tang, Y. and Toews, D. P. (1990). Determination of intracellular pH and PCO2 values after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* **81**, 255–274.

Rahn, H., Rahn ,K. B., Howell, B. J., Gans, C. and Tenney, S. M. (1971) Air breathing of the garfish (*Lepisosteus osseus*). *Respir. Physiol.* **11**, 285–307.

Rahn, H. (1966). Aquatic gas exchange: theory. *Respir. Physiol.* **1**, 1-12.

Rainboth, W. J. (1996). Fishes of Cambodian Mekong. FAO speices indentification field guide for fishery purposes. FAO, Rome.

Randall, D. J., Cameron, J. N. (1973). Respiratory control of arterial pH as temperature changes in rainbow trout *Salmon gairdneri*. *Am. J. Physiol.* **225**, 997–1002.

Reeves, R. B. (1972). An imidazole alphastat hypothesis for vertebrate acid-base regulation: Tissue carbon dioxide content and body temperature in bullfrogs. *Respir. Physiol.* **14**, 219–236.

Reeves, R. B. (1977). The interaction of body temperature and acid-base balance in ectothermic vertebrates. *Annu. Rev. Physiol.* **39**, 559-586.

Shafland, P.L., Gestring, K.B. and Stanford, M.S. (2009). An assessment of the Asian swamp eel (*Monopterus albus*) in Florida. *Reviews in Fisheries Science* **18**, 25–39.

Shartau, R. B. and Brauner, C.J. (2014) Acid–base and ion balance in fishes with bimodal respiration. *J. Fish. Biol.* **84**, 682–704.

Shih, H. J. (1940). On the foods of *Monopterus*. *Sinensia* **11**, 573-576.

Smatresk, N. J. and Cameron, J.N. (1982) Respiration and acid-base physiology of the spotted gar, a bimodal breather: II. Responses to temperature change and hypercapnia. *J. Exp. Biol.* **96**, 281–293

Stewart, P.A. (1978). Independent and dependent variables of acid-base control. *Respir. Physiol.* **33**, 9-26

Stinner, J. N. and Hartzler, L. K. (2000). Effect of temperature on pH and electrolyte concentration in air-breathing ectotherms. *J. Exp. Biol.* **203**, 2065-2074

Truchot, J. P. (1987). *Comparative Aspects of Extracellular Acid–Base Balance*. Springer-Verlag, Berlin.

Ultsch, G. R. and Jackson, D. C., (1996). *pH and temperature in ectothermic vertebrates*. Bull. Alabama Mus. Nat. Hist

Walsh, P. J. and Moon, T.W. (1982). The influence of temperature on extracellular and intracellular pH in the Americal eel, *Anguilla rostrata* (Le Sueur). *Respir. Physiol.* **50**, 129-140

Wang, T., Abe, A. S. and Glass, M. L. (1998). Temperature effects on lung and blood gases in *Bufo paracnemis*: consequences of bimodal gas exchange. *Respir. Physiol.* **113**, 231-238.

Wang, T., Abe A. S. and Glass, M. L. (1998). Effects of temperature on lung and blood gases in the South American rattlesnake *Crotalus durissus terrificus*. *Comp. Biochem. Physiol.* **121A**, 7–11.

Wang, T. and Jackson, D. C. (2016). How and why pH changes with body temperature: the α-stat hypothesis. *J. Exp. Biol.* **219**, 1090–1092.

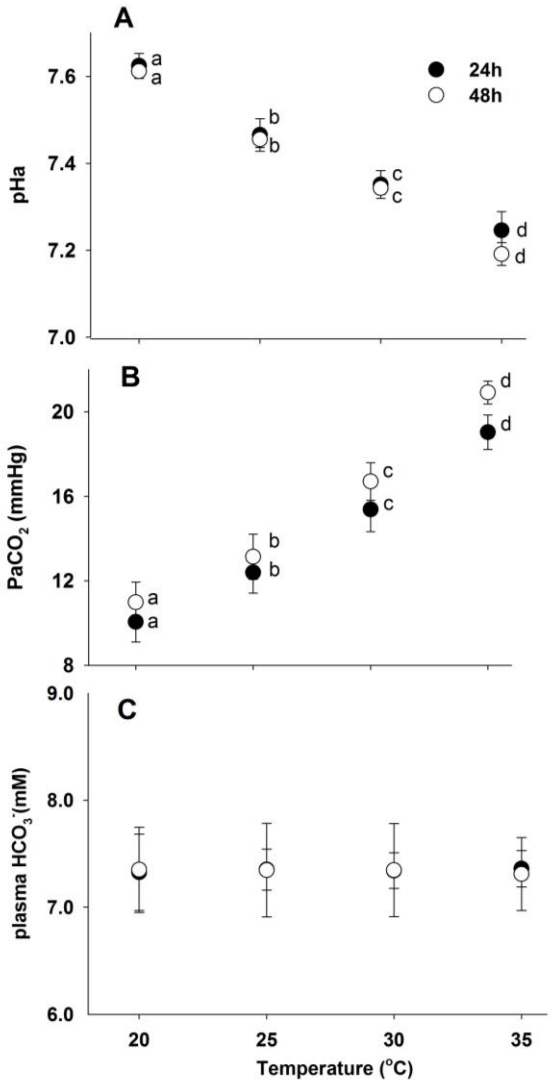

Fig. 1. **Effects of elevated temperature on arterial blood acid-base status.** Arterial pH (A), partial pressure of carbon dioxide (B), and plasma [HCO_3^-] (C) in cannulated *Monopterus albus* held at different temperatures for 24h (closed circles) and 48h (open circles). Within each panel, letters that differ indicate statistically significant differences *($p<0.05$)*. A one-way ANOVA was used for comparison between difference temperatures in a given time. Values are mean ± S.E.M. *($N=6$)*.

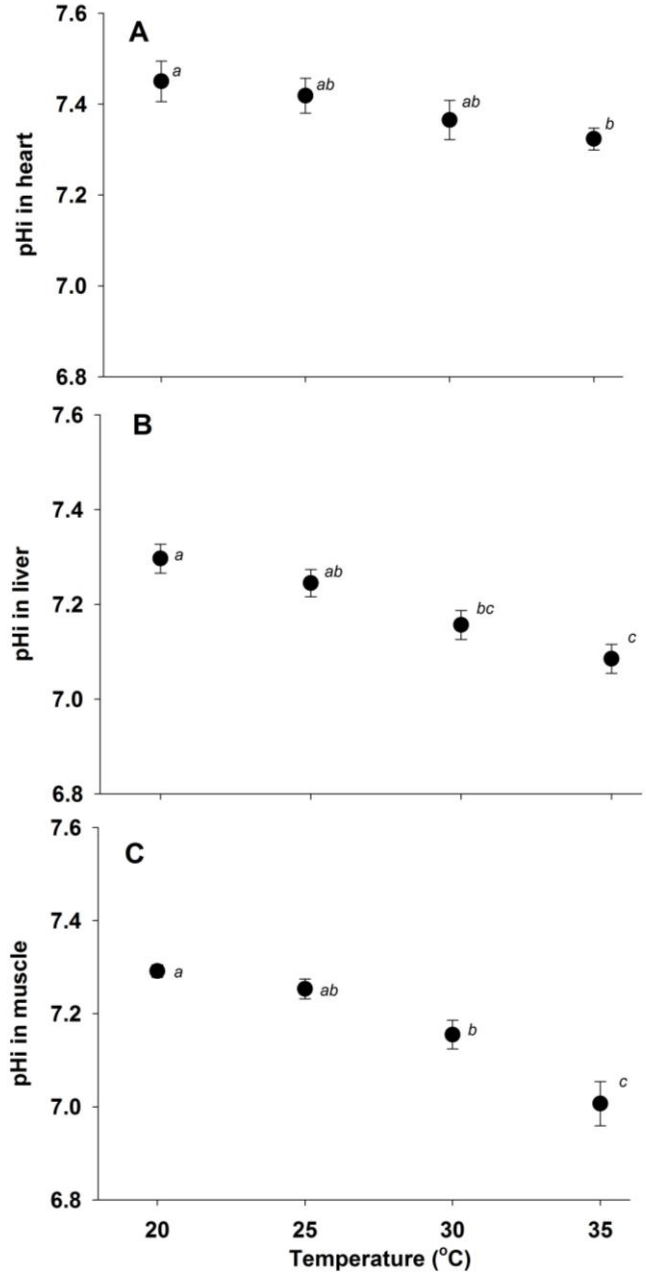

Fig 2. Effects of elevated temperature on intracellular pH. Intracellular pH in heart (A), liver (B) and muscle (C) of *Monopterus albus* held at 20, 25, 30 or 35 C for 24 h. Letters that differ within a panel indicate statistically significant differences (p<0.05). A one-way ANOVA was used for comparison between difference temperatures in a given time. Values are mean ± S.E.M. *(N=5).*

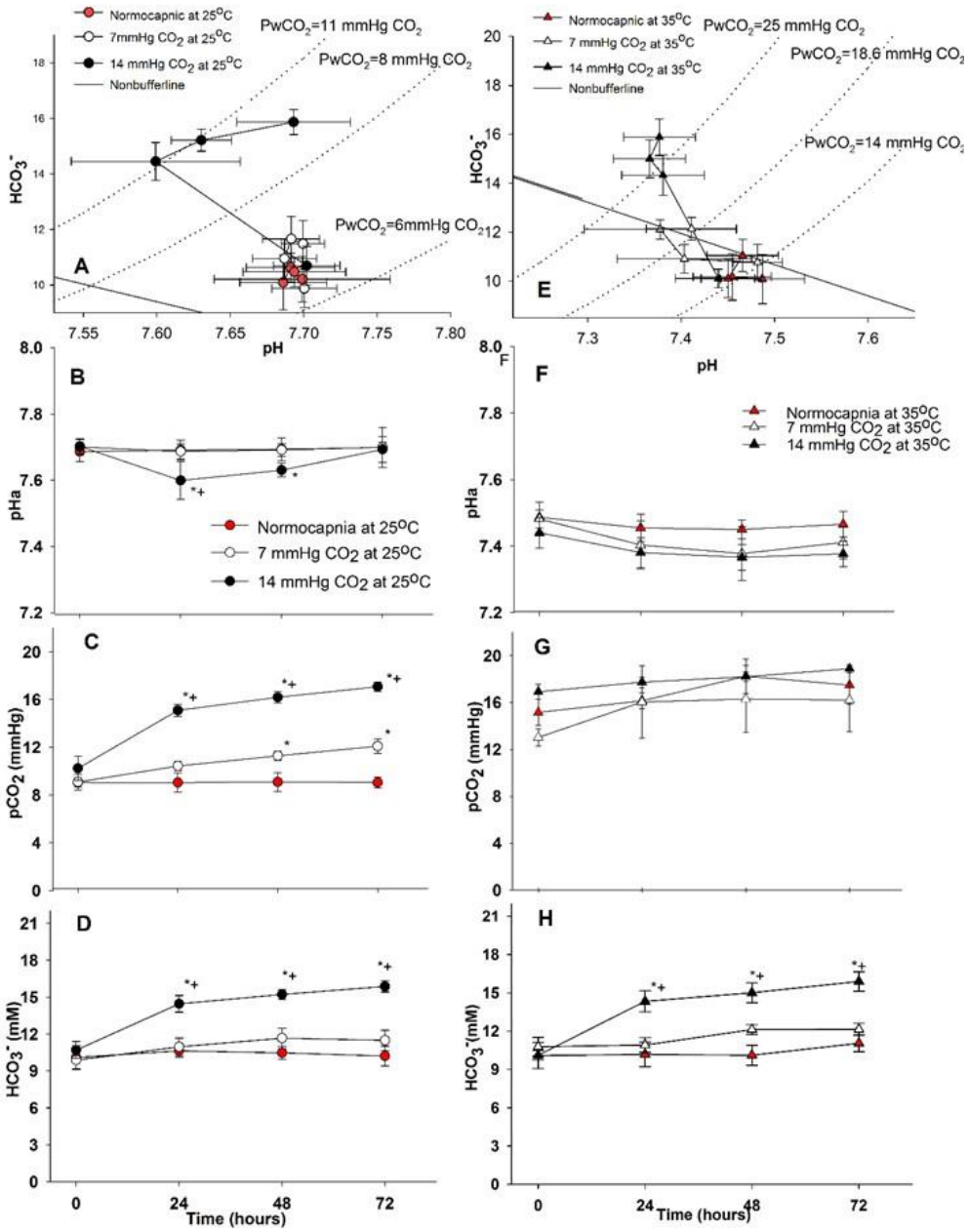

Figure 3. Effects of temperature on the metabolic compensation of arterial pH. Davenport diagram with CO_2 isopleths at $PaCO_2$ upon exposure to 7 mmHg CO_2 and 14 mmHg CO_2 at 25°C (A) and 35°C (B). Arterial plasma pH (pHe) (B, F), partial pressure of CO2 (C, G), and [HCO3−] (D, H) after exposure to normocapnia (red circle), or a PCO2 of 7 (open circle) or 14 mmHg CO2 (closed circle) at 25°C (left panels, i.e. A, B and C) or 35°C (right panels, i.e. D, E and F). Asterisks indicate statistically significant differences from day 0 at a given PCO2 and "+" indicates a statistically significant difference from the normocapnia at the respective sampling time. A two-way ANOVA was used for comparison between difference temperatures in a given time. Values are means±s.e.m *(N=6).*

Table 1. Effects of elevated temperature on arterial plasma ion concentration. Arterial plasma Na^+, K^+, Cl^- concentrations and osmolality from cannulated *Monopterus albus* following 24h and 48h at 20, 25, 30 and 35°C. *Letters that differ within a column indicate statistically significant differences ($p<0.05$).* A one-way ANOVA was used for comparison between difference temperatures in a given time. Values are mean ± S.E.M. *($N=6$).*

Temperature (°C)	Na^+ (mM)		K^+ (mM)		Cl^- (mM)		Osm (mOsm)	
	24h	48h	24h	48h	24h	48h	24h	48h
20	123.5±1.3[a]	123.3±1.2[a]	2.8±0.2[a]	2.9±0.2[a]	111.1±3.1[a]	112.0±2.2[a]	287.3±3. 1[a]	290.8±3.8[a]
25	123.1±1.8[a]	122.3±1.8[a]	2.9±0.2[a]	2.8±0.1[a]	111.2±4.0[a]	110.8±2.5[a]	290.0±2.5[a]	287.2±2.5[a]
30	124.4±1.6[a]	122.7±2.2[a]	2.9±0.2[a]	2.8±0.2[a]	111.8±2.0[a]	112.8±2.1[a]	287.2±3.5[a]	288.0±5.2[a]
35	123.3±1.7[a]	122.4±1.3[a]	3.3±0.1[a]	3.5±0.1[b]	112.8±3.5[a]	112.0±4.3[a]	287.0±2.3[a]	288.1±3[a]

Table 2. Effects of elevated temperature on arterial blood haemoglobin concentration and hematocrit. Arterial blood haemoglobin concentration (mM) and haematocrit (%) in cannulated *Monopterus albus* following 24h and 48h at 20, 25, 30 and 35°C. *Letters that differ within a column indicate statistically significant differences ($p<0.05$).* A two-way ANOVA was used for comparison between difference temperatures in a given time. Values are mean ± S.E.M. *($N=6$).*

Temp (°C)	[Hb] (mM)		Hct (%)	
	24h	48h	24h	48h
20	8.1 ± 0.1[a]	8.4 ± 0.1[a]	51.9 ± 0.2[a]	52.5 ± 0.3[a]
25	8.3 ± 0.1[a]	8.3 ± 0.1[a]	52.2 ± 0.3[a]	52.7 ± 0.3[a]
30	8.4 ± 0.1[a]	8.3 ± 0.1[a]	53.7 ± 0.3[a]	53.9 ± 0.3[a]
35	8.8 ± 0.1[a]	8.8 ± 0.1[a]	54.4 ± 0.2[a]	55.4 ± 0.2[a]

Table 3

Effects of combined temperature and hypercapnia on arterial plasma ion concentration

Arterial plasma Na^+, K^+, Cl^- concentration and osmolality in cannulated *Monopterus albus* after exposure to normocapnia or a PCO_2 of 7 or 14 mmHg CO_2 at 25°C or 35°C. *Two-way ANOVA was used for comparison between difference temperature in a given time and data are mean* ± S.E.M. *(N=6).*

Na^+ (mM)	**0 h**	**24 h**	**48 h**	**72 h**
25°C Normocapnia	139.8±1.6	140.2±1.5	140.7±2.4	139.3±1.3
25°C 7 mmHg CO_2	138.7±1.2	138.5±1	138.2±1.1	138.2±1.0
25°C 14 mmHg CO_2	139.6±1.7	138.8±1.8	138.5±1.4	137.9±1.4
35°C Normocapnia	139.5±0.9	139.6±1	139.4±1.0	139.3±1.5
35°C 7 mmHg CO_2	140.7±1.4	139.6±2.1	138.4±1.2	137.0±0.9
35°C 14 mmHg CO_2	138.9±1.4	137.1±1.4	136.8±1.2	136.2±1.0

K^+ (mM)	**0 h**	**24 h**	**48 h**	**72 h**
25°C Normocapnia	2.9±0.4	2.9±0.2	3.0±0.4	3.0±0.4
25°C 7 mmHg CO_2	3.0±0.2	3.1±0.1	3.4±0.3	3.6±0.3
25°C 14 mmHg CO_2	3.4±0.4	3.3±0.3	3.4±0.2	3.4±0.2
35°C Normocapnia	3.2±0.4	3.1±0.2	3.2±0.4	3.5±0.3
35°C 7 mmHg CO_2	3.2±0.3	3.5±0.6	3.4±0.2	3.3±0.6
35°C 14 mmHg CO_2	2.5±0.6	3.4±0.3	3.4±0.3	3.5±0.4

Cl^- (mM)	**0 h**	**24 h**	**48 h**	**72 h**
25°C Normocapnia	112.4±3.8	111.6±1.9	112.4±2.0	112.4±2.3
25°C 7 mmHg CO_2	112.0±3.6	111.2±1.2	112.1±1.6	111.9±2.4
25°C 14 mmHg CO_2	111.9±0.8	110.2±1.5	109.9±0.4	109.7±0.9
35°C Normocapnia	112.0±1.4	112.2±2.2	112.3±2.3	111.8±2.0
35°C 7 mmHg CO_2	112.0±2.4	111.4±1.2	110.9±2.0	110.8±1.2
35°C 14 mmHg CO_2	112.2±2.2	110.6±1.4	109.7±0.7	109.7±1.1

Osm (mOsm)	**0 h**	**24 h**	**48 h**	**72 h**
25°C Normocapnia	288.7±3.8	288.3±3.6	288.5±2.1	288.3±2.0
25°C 7 mmHg CO_2	288.5±3.3	288.1±3.3	288.3±3.1	288.1±1.5
25°C 14 mmHg CO_2	288.3±2.8	286.3±2.9	285.5±2.1	286.1±3.4
35°C Normocapnia	289.6±1.0	289.5±1.2	289.3±1.0	289.5±1.3
35°C 7 mmHg CO_2	290.6±2.9	289.3±4.5	288.5±2.9	288.0±2.3
35°C 14 mmHg CO_2	288.6±2.9	286.3±2.7	286.1±2.2	286.1±3.3

Table 4. **Effects of combined temperature and hypercapnia on arterial blood haemoglobin concentration and hematocrit**. Arterial blood haemoglobin concentration (mM) and hematocrit (%) from cannulated *Monopterus* after exposure to normocapnia, or a PCO_2 of 7 or 14 mmHg at 25°C or 35°C. *Two-way ANOVA was used for comparison between difference temperature in a given time and data are mean* ± S.E.M. *(N=6).*

[Hb] (mM)	**0 h**	**24 h**	**48 h**	**72 h**
25°C Normocapnia	8.5±0.8	8.3±0.4	8.5±0.7	8.2±0.5
25°C 7 mmHg CO_2	8.3±0.3	8.4±0.3	8.3±0.3	8.3±0.4
25°C 14 mmHg CO_2	8.4±0.2	8.3±0.2	8.4±0.2	8.3±0.2
35°C Normocapnia	8.5±0.3	8.6±0.4	8.5±0.5	8.6±0.2
35°C 7 mmHg CO_2	8.4±0.3	8.6±0.4	8.6±0.3	8.7±0.2
35°C 14 mmHg CO_2	8.5±0.3	8.6±0.2	8.7±0.3	8.7±0.2

Hct (%)	**0 h**	**24 h**	**48 h**	**72 h**
25°C Normocapnia	49.6±1.5	49.5±1.3	49.4±0.7	49.5±1.6
25°C 7mmHg CO_2	50.3±0.5	49.9±1.0	49.8±1.0	49.1±2
25°C 14mmHg CO_2	50. 5±0.4	50.0±0.4	49.6±0.5	49.2±0.4
35°C Normocapnia	51.7±1.6	51.1±1	51.8±1	52.1±1.9
35°C 7 mmHg CO_2	52±1.4	51.0±1.3	51.4±1.3	51.4±1.8
35°C 14 mmHg CO_2	50.5±0.5	51±0.5	51. 5±0.7	51.8±0.7

Table 5. Comparison of extra and intracellular pH changes with temperature (ΔpH/Δ°C) in some fish, amphibians and reptiles for comparison with values obtained in this study.

Species	Temp (°C)	ΔpH/Δ°C				
		Blood	***White muscle***	***Heart***	***Red muscle***	***Liver***
Frog (*Rana catesbeiana*)[1]	3.5-30	-0.0204	-0.0152			
Turtle (*Pseudemys scripta*)[2]	9-32	-0.021	-0.014			-0.023
Dogfish (*Scyloirhinus stellaris*)[3]	10-23	-0.0148	-0.0178	-0.0098	-0.0334	
American eel (*Aguilia rostrata*)[4]	5-20	-0.0076	-0.009	-0.0205	-0.0033	-0.0177
Channa catfish (*Ictalurus punctatus*)[5]	15-31	-0.0132	-0.015	-0.012	-0.018	
Anuran amphibians (*Xenopus Laevis*)[6]	10-30	-0.017		-0.007	-0.017	
Anuran amphibians (*Bufo marinus*)[7]	10-30	-0.015		-0.026	-0.023	
Swamp eel (*Monopterus albus*)[8]	**20-35**	**-0.025**	**-0.019**	**-0.0084**		**-0.014**

[1,2]Malan et al., 1976; [3]Heisler & Weitz, 1976; [4]Walsh & Moon, 1982; [5]Cameron & Kormanik, 1982; [6,7]Boutilier & Heisler, 1987 and [8]this study