

## SHORT COMMUNICATION

# Effects of temperature on acid–base regulation, gill ventilation and air breathing in the clown knifefish, *Chitala ornata*

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## ABSTRACT

*Chitala ornata* is a facultative air-breathing fish, which at low temperatures shows an arterial  $P_{\text{CO}_2}$  ( $P_{\text{aCO}_2}$ ) level only slightly elevated above that of water breathers. By holding fish with indwelling catheters at temperatures from 25 to 36°C and measuring blood gasses, we show that this animal follows the ubiquitous poikilotherm pattern of reducing arterial pH with increasing temperature. Surprisingly, the temperature increase caused an elevation of  $P_{\text{aCO}_2}$  from 5 to 12 mmHg while the plasma bicarbonate concentration remained constant at around 8 mmol l<sup>-1</sup>. The temperature increase also gave rise to a larger fractional increase in air breathing than in gill ventilation frequency. These findings suggest that air breathing, and hence the partitioning of gas exchange, is to some extent regulated by acid–base status in air-breathing fish and that these bimodal breathers will be increasingly likely to adopt respiratory pH control as temperature rises, providing an interesting avenue for future research.

**KEY WORDS:** Gas exchange partitioning, Air-breathing fish, pH regulation

## INTRODUCTION

The vast majority of ectothermic vertebrates reduce arterial pH (pHa) as body temperature increases (Amin-Naves et al., 2007; Damsgaard et al., 2018; Fobian et al., 2014; Heisler, 1984; Rahn et al., 1971; Reeves, 1977; Smatresk and Cameron, 1982; Thinh et al., 2018; Truchot, 1987; Ultsch and Jackson, 1996; Wang and Jackson, 2016). Aquatic water-breathing vertebrates are constrained to achieve pHa adjustments through modulation of plasma  $\text{HCO}_3^-$  concentration ( $[\text{HCO}_3^-]$ ) because their low arterial  $P_{\text{CO}_2}$  ( $P_{\text{aCO}_2}$ ) demands large ventilatory changes to adjust pHa, potentially compromising oxygen uptake given the low oxygen solubility in water (Bayley et al., 2019; Dejours, 1981; Rahn, 1966). In contrast, terrestrial vertebrates with their higher  $P_{\text{aCO}_2}$  (Rahn, 1966) can rapidly regulate blood pH across temperatures through small changes in pulmonary ventilation (Glass and Wood, 1983; Jackson, 1989). This has the additional advantage of avoiding the osmotic changes that can ensue from the epithelial ion exchange adopted by aquatic animals (Austin et al., 1927; Burton, 2002). The transition from water to air breathing was also accompanied by

a shift from primarily regulating arterial oxygen levels in water breathers by virtue of peripheral receptors to central monitoring of  $\text{CO}_2$  and pH in air breathers (Bayley et al., 2019; Milsom, 2002, 2010; Milsom and Burleson, 2007).

There are approximately 400 species of bimodally breathing fish, which are extant evidence of the vertebrate transition from aquatic to terrestrial life, and air breathing has independently arisen on >80 occasions throughout the vertebrate phylogeny (Damsgaard et al., 2019). Air-breathing species give fascinating insight into the transitional states and are divided into obligate air breathers, which drown if prevented access to air, and facultative air breathers, which thrive in normoxic water without air breathing.  $P_{\text{aCO}_2}$  in these two groups reflects their breathing habits (Rahn, 1966), with  $P_{\text{aCO}_2}$  of facultative fish being similar to that of water breathers, whereas the higher  $P_{\text{aCO}_2}$  of obligate air-breathing fish resembles that of tetrapods (Bayley et al., 2019). It might therefore be hypothesized that the modality used to adjust pHa across environmental temperature should follow the animal's partitioning pattern for gas exchange across the air–water interface. Temperature itself influences partitioning, with an increased reliance on the air phase as temperature increases associated with a concomitant elevation of  $P_{\text{aCO}_2}$  in air-breathing garfishes (*Lepisosteus oseus* and *Lepisosteus oculatus*) (Rahn et al., 1971; Smatresk and Cameron, 1982). Furthermore, the facultative air-breathing striped catfish (*Pangasionodon hypophthalmus*) shows the aquatic vertebrate-type temperature adjustment by modulating plasma  $[\text{HCO}_3^-]$  when in normoxic water (Damsgaard et al., 2018), but modulates  $P_{\text{aCO}_2}$  across temperature in hypoxic water, where this species relies on aerial gas exchange. Finally, the obligate swamp eel (*Monopterus albus*) with vestigial gills and a tetrapod-like  $P_{\text{aCO}_2}$  seems to regulate pHa by ventilatory adjustments while plasma  $[\text{HCO}_3^-]$  is unaffected by temperature (Thinh et al., 2018).

The air-breathing clown knifefish (*Chitala ornata* Gray 1831) is commercially grown across South-East Asia, where it is favoured both as an ornamental fish and as a source of protein for human consumption. It regulates pHa when confronted with aquatic hypercapnia (Gam et al., 2018) – something that has been considered unusual in air-breathing fish, which typically show a reduced gill surface area, argued to limit transepithelial ion exchange and therefore branchial capacity for extracellular acid–base regulation (Brauner and Baker, 2009; Shartau and Brauner, 2014). Further, it has recently found that, unique among teleosts, it possesses a strong ventilatory response to changes in  $P_{\text{aCO}_2}$  and is thus argued to have internally orientated  $\text{CO}_2/\text{H}^+$  sensors (Tuong et al., 2018a; Tuong et al., 2019). However, it is a facultative air breather (Tuong et al., 2018b) with a  $P_{\text{aCO}_2}$  of 5–8 mmHg in normoxic normocapnic water (Gam et al., 2018). These aspects make it an interesting object to study the regulation of pHa with temperature changes, something that has also become timely with global warming scenarios, where median temperature in its habitat

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**List of abbreviations**

[Hb]	haemoglobin concentration
[HCO <sub>3</sub> <sup>-</sup> ]	plasma bicarbonate concentration
Hct	haematocrit
MCHC	mean cellular haemoglobin concentration
<i>P</i> <sub>CO<sub>2</sub></sub>	partial pressure of CO <sub>2</sub> in arterial blood
<i>P</i> <sub>O<sub>2</sub></sub>	partial pressure of O <sub>2</sub> in arterial blood
pHa	pH in arterial blood
<i>pK'</i>	apparent <i>pK</i> value for the CO <sub>2</sub> to HCO <sub>3</sub> <sup>-</sup> reaction
TCO <sub>2</sub>	total CO <sub>2</sub> of plasma

is expected to increase from the current 27–29°C (Li et al., 2013) to as much as 33°C within the next century (IPCC, 2014).

Here, we tested the hypothesis that *C. ornata*, with its ventilatory response to *P*<sub>CO<sub>2</sub></sub>, follows the pattern of acid–base regulation across temperature seen in terrestrial amniotes, with constant [HCO<sub>3</sub><sup>-</sup>] and modulated *P*<sub>CO<sub>2</sub></sub>. We assessed this by measuring arterial blood gases and relevant plasma ions in chronically catheterized animals across relevant environmental temperatures as well as measuring gill- and air-breathing ventilation rates from 24 to 36°C. Further, we (Thinh et al., 2018) previously proposed that a way of confirming the deliberate temperature-induced regulation of plasma pH would be to expose the animal to environmental hypercapnia at the temperature extremes and determine whether the animal defended the temperature-specific pH set-point. Therefore, we also exposed animals acclimated to 25 and 33°C to aquatic hypercapnia (21 mmHg CO<sub>2</sub>) and followed acid–base status over the subsequent 72 h.

**MATERIALS AND METHODS****Experimental holding**

Clown knifefish (*C. ornata*, mean±s.e.m. body mass 503±55 g) were purchased from a local aquaculture farm, transported to Can Tho University and held in 4 m<sup>3</sup> tanks at 27°C with constant aeration (dissolved oxygen >90%, *P*<sub>CO<sub>2</sub></sub><0.7 mmHg and pH 7.75–7.85) for 2 weeks prior to experimentation. Fish were fed commercial shrimp feed with 38% protein (Tomboy Aquafeed Company, Vietnam). Water was changed (30%) every second day and total ammonia and nitrite were always below 40 µmol l<sup>-1</sup> and 1 µmol l<sup>-1</sup>, respectively. Fish were fasted for 24 h prior to cannulation and throughout experimentation. All experiments were conducted in accordance with national guidelines on animal welfare in Vietnam.

**Animal dorsal aorta catheterization**

Fish were anaesthetized in 0.05 g l<sup>-1</sup> benzocaine and moved to a surgical table where the gills were irrigated with water containing 0.025 g l<sup>-1</sup> benzocaine while a polyethylene PE40 catheter was inserted into the dorsal aorta through the dorsal surface of the buccal cavity (Soivio et al., 1975). The catheter was filled with heparinized saline (50 IU per 1 ml saline) and fixed with several stitches on the mouth, nose and near the dorsal fin. Fish were allowed to recover in well-aerated water for 24 h at 27°C before experimentation to normalize blood gasses and lactate levels (Phuong et al., 2017a).

**Measurements of ventilation rate**

Additional fish were anaesthetized as described above for attachment of impedance leads to the outer surface of both the left and right opercula and secured in place with nylon suture. This procedure lasted a maximum of 10 min. Fish were allowed to recover in well-aerated 27°C water for 24 h prior to

experimentation, at which time the leads were connected to the impedance converter via an acquisition system (Dataq DI-720, DATAQ Instruments, Inc., Akron, OH, USA) recording at 125 Hz for ventilatory measurements.

**Experimental design****Series 1: effects of temperature on arterial acid–base status**

Four temperature levels were tested in the experiment (25, 30, 33 and 36°C), each replicated 6 times. In addition, as the entire procedure was identical to one used previously (Gam et al., 2018), we included the dataset for 27°C from that study in the present analysis. Following surgical recovery at the standard laboratory temperature at Can Tho of 27°C, each replicate was moved to separate 120 l tanks containing water circulated from a central 500 l tank. Temperature was elevated (or decreased) at 0.5°C h<sup>-1</sup> to the target temperature and left for 48 h. Thereafter, blood was drawn at 0, 24, 48 and 72 h from the dorsal aorta catheter into heparinized syringes. Acid–base parameters [pHa, *P*<sub>CO<sub>2</sub></sub>, total CO<sub>2</sub> of plasma (TCO<sub>2</sub>)] were measured immediately. These values were used to calculate the temperature-specific acid dissociation constant (*pK'*). In the same blood samples, haemoglobin concentration ([Hb]) and haematocrit (Hct) were measured. Remaining blood was centrifuged and the plasma stored at –80°C for subsequent analysis of ions (Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>) and osmolality.

**Series 2: effects of temperature on gill ventilation and air-breathing frequency**

After they had recovered from the insertion of impedance electrodes, fish were moved individually to the temperature system described above. Water temperature was progressively increased as in series 1. After 48 h at the target temperature, fish were moved to a 50 l glass tank containing fresh target temperature water and left for 1 h to reach resting levels; gill ventilation and air-breathing frequency were then measured for 60 min.

**Series 3: effects of temperature and hypercapnia on pHa regulation**

A total of 24 fish were used. After recovery from cannulation, fish were transferred to the system described in series 1. An Oxyguard Pacific system coupled with a G10 ps CO<sub>2</sub> probe and a K01 svpld pH probe (Oxyguard International A/S, Farum, Denmark) and a solenoid valve was used to regulate water *P*<sub>CO<sub>2</sub></sub> at 21 mmHg. In the normocapnic treatment, *P*<sub>CO<sub>2</sub></sub> was kept below 0.7 mmHg, the lower detection limit of the system, by vigorous aeration. This experiment was performed at both 25 and 33°C. Temperature was adjusted as in series 1 and blood subsequently sampled at 0, 24, 48 and 72 h for measurement of the same blood parameters.

**Analytical methods**

The non-bicarbonate buffer line and temperature-specific *pK'* values were calculated from data from series 1. pHa and *P*<sub>CO<sub>2</sub></sub> were measured using an iSTAT analyser (iSTAT Corporation, Princeton, NJ, USA) with CG3+ cartridges and temperature corrected using the iSTAT manual equation as validated previously (Gam et al., 2018). TCO<sub>2</sub> was measured using a Cameron chamber, where a blood aliquot is added to a known acid volume and the resulting elevation in *P*<sub>CO<sub>2</sub></sub> reflects the total CO<sub>2</sub> content of the sample (Cameron, 1971). [HCO<sub>3</sub><sup>-</sup>] was calculated using the equation: [HCO<sub>3</sub><sup>-</sup>]=TCO<sub>2</sub>–*P*<sub>CO<sub>2</sub></sub>×αCO<sub>2</sub>, where αCO<sub>2</sub> is CO<sub>2</sub> solubility in trout plasma (Boutilier et al., 1984).

Plasma Cl<sup>-</sup> was measured using a chloride titrator (Sherwood, model 926S MK II Chloride analyser, Sherwood Science Ltd, Cambridge, UK) and plasma Na<sup>+</sup> and K<sup>+</sup> with flame photometry

(Sherwood model 420). Total plasma osmolality was measured on a Fiske One-Ten Micro Osmometer (Fiske\*Associates, Two Technology Way, Norwood, MA, USA).

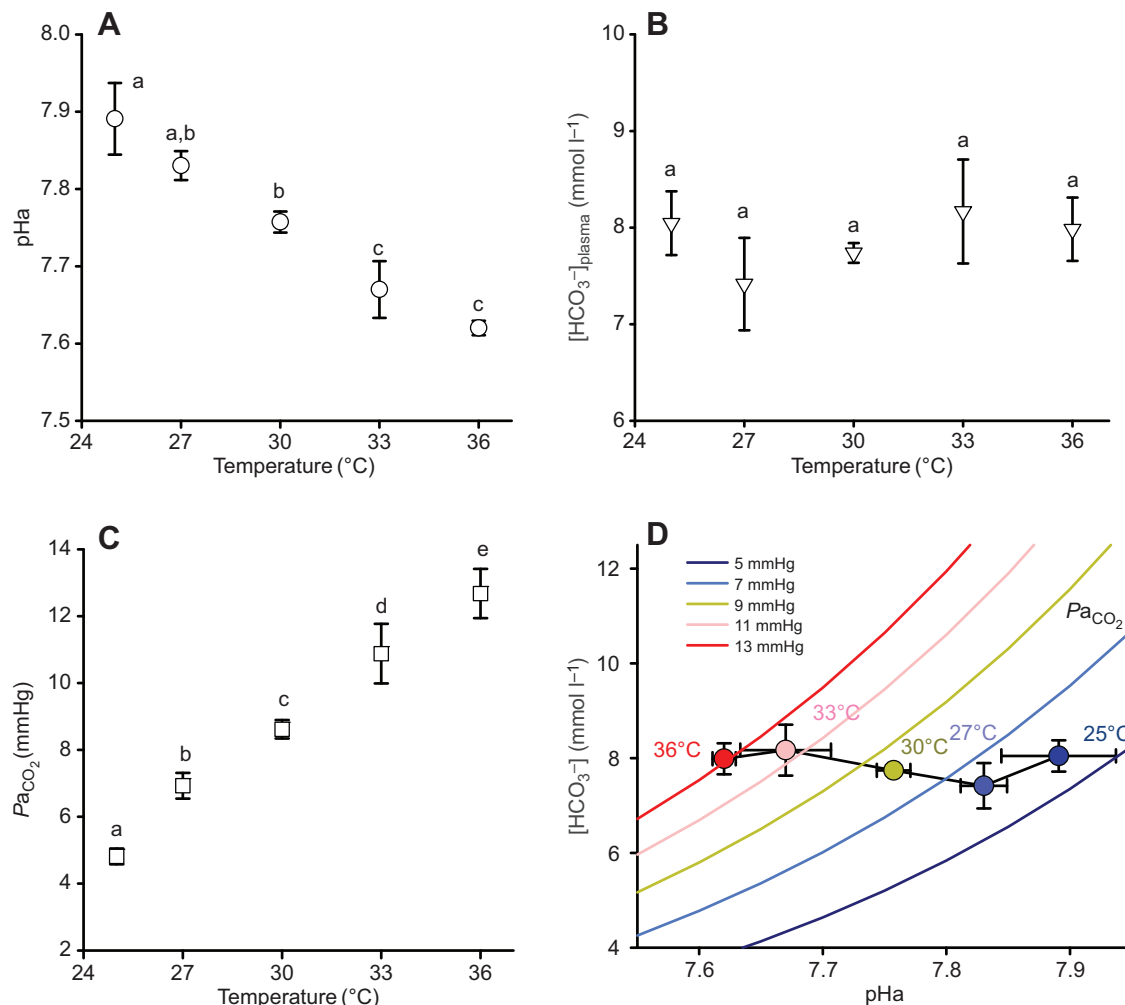
Hct was measured as a fractional volume of packed red blood cells after centrifugation at 12,000 *g* for 3 min. [Hb] was spectrophotometrically measured at 540 nm after conversion to cyano-methaemoglobin using Drabkin's solution (Zijlstra et al., 1983). Mean corpuscular haemoglobin concentration (MCHC) was calculated from the ratio between [Hb] and Hct.

### Statistics

All figures were made in Sigma plot 12.5 and data were analysed with PASW statistics (SPSS18.0). A one-way ANOVA was used to evaluate the effects of temperature on arterial acid–base parameters (series 1). A two-way ANOVA (the Holm–Šidák multiple comparison method, pair-wise comparison) was used to identify differences between sampling time and treatments to evaluate the effects of temperature on gill ventilation and air-breathing frequency and the effects of temperature and hypercapnia on pH<sub>a</sub> regulation (series 2 and 3). A *P*-value of less than 5% (*P*<0.05) was considered significant. All data are shown as means±s.e.m.

### RESULTS AND DISCUSSION

The pattern of pH<sub>a</sub> adjustment across environmental temperature shown by *C. ornata* resembles that of terrestrial vertebrates with large changes in  $P_{aCO_2}$ , but without the typical piscine adjustments in plasma  $[HCO_3^-]$  (Fig. 1). The slope of the pH<sub>a</sub> change of  $-0.024$  pH units  $^{\circ}C^{-1}$  is higher than that in most other vertebrates, but by no means unique. The air-breathing rice eel, *M. albus*, showed a similar slope of  $-0.025$  pH units  $^{\circ}C^{-1}$  (Thinh et al., 2018) and examination of values across vertebrates reveals several other similar examples (Ultsch and Jackson, 1996). Plasma  $[Na^+]$ ,  $[K^+]$ ,  $[Cl^-]$  and osmolality remained largely unaffected across temperature (Table S1). A slight but significant reduction in [Hb] and Hct was seen with temperature elevation, due probably to repeated blood sampling, while MCHC remained unchanged (Table S2). A similar pattern with elevated  $P_{aCO_2}$  has been observed in two other air-breathing teleosts, *P. hypophthalmus* and *M. albus* (Damsgaard et al., 2018; Thinh et al., 2018). Both *P. hypophthalmus* and *C. ornata* are facultative air breathers, relying predominantly on their gills for oxygen uptake in normoxic water (Lefevre et al., 2011a, 2013; Tuong et al., 2018b; Ultsch and Jackson, 1996). While *P. hypophthalmus* only showed this pattern

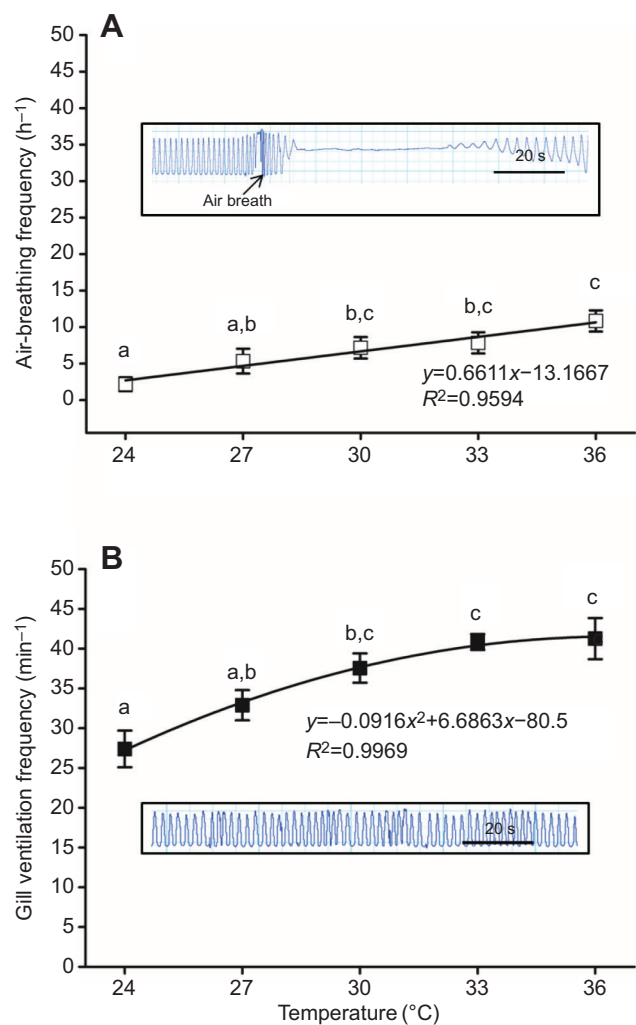


**Fig. 1. Effects of elevated temperature on acid–base status in arterial blood.** (A) Arterial pH (pH<sub>a</sub>). (B) Arterial partial pressure of carbon dioxide ( $P_{aCO_2}$ ). (C) Plasma  $HCO_3^-$ . (D) Davenport diagram with temperature-specific  $P_{aCO_2}$  isopleths after 72 h exposure to elevated temperatures: 25, 27, 30, 33 and 36°C. The  $P_{aCO_2}$  isopleths are colour coded to match the temperature indicated by text in the same colour, and were calculated using the appropriate temperature-adjusted constants. The data at 27°C were obtained from Gam et al. (2018). A one-way ANOVA was used for comparison between different temperatures. Different letters indicated significant differences (*P*<0.05). Data are means±s.e.m. (*N*=6).

in hypoxic water, resembling a classic water breather in normoxia, the obligate air breather *M. albus* modulated  $P_{aCO_2}$  even in normoxic water (Thinh et al., 2018).

Changing the respiratory medium significantly impacts blood gases and a shift to air breathing brings about a rise in blood  $P_{aCO_2}$  through the lowering of ventilation rates when fish transition to air breathing (Rahn et al., 1971). This elevated  $P_{aCO_2}$  allows for ventilatory regulation of plasma pH, because at high  $P_{aCO_2}$ , small changes in ventilation give rise to large changes in pH, whereas at the low  $P_{aCO_2}$  levels of water breathers, elevation of  $P_{aCO_2}$  requires significant ventilatory changes that are likely to impact oxygen uptake (Bayley et al., 2019). Indeed, there is one water-breathing fish that illustrates this dilemma (*Piaractus mesopotamicus*) because it regulates arterial pH across temperature by increasing  $P_{aCO_2}$  at the expense of arterial  $P_{O_2}$  ( $P_{aO_2}$ ) (Soncini and Glass, 1997). The authors argue for this species that the drop in  $P_{aO_2}$  is of little consequence, because a right shift in the haemoglobin–oxygen binding curve facilitates tissue unloading and the reduced  $P_{aO_2}$  remains on the upper flat part of the blood equilibrium curve, hence only marginally affecting saturation. Air-breathing fish in general show  $P_{aCO_2}$  values that clearly reflect the partitioning of  $CO_2$  excretion, with those predominantly using their gills showing levels similar to those of pure water breathers, while those reliant on air breathing showing  $P_{aCO_2}$  levels between 14 and 28 mmHg (Bayley et al., 2019; Thinh et al., 2018). Hence, the ventilatory adjustments required to alter pH<sub>a</sub> in response to temperature in the obligate *M. albus* (Thinh et al., 2018) are relatively minor. *Pangasionodon hypophthalmus*, in contrast, is a facultative air breather with little or no aerial gas exchange in normoxic water (Lefevre et al., 2011a, 2013), low  $P_{aCO_2}$  levels (Damsgaard et al., 2015) and no apparent ventilatory response to even very severe aquatic hypercapnia (Thomsen et al., 2017). Temperature-induced adjustments of pH<sub>a</sub> in normoxic water thus follow the standard aquatic organism pattern, with transmembrane ion exchange and little change in  $P_{aCO_2}$ , whereas in hypoxia,  $P_{aCO_2}$  is elevated as a result of increased air breathing, leaving only minor adjustments required through branchial  $HCO_3^-/Cl^-$  exchange (Damsgaard et al., 2018).

In *C. ornata*, increased temperature stimulated both air breathing and gill ventilation (Fig. 2), but the  $Q_{10}$  for gill ventilation was considerably lower than that for air breathing (1.4 and 3.8, respectively), indicating a progressive transition from aquatic to aerial gas exchange as temperature rose. While *C. ornata* is a facultative air breather, a mere 86% of its oxygen is covered by uptake over its small branchial surfaces in normoxic water at 27°C (Tuong et al., 2018b), whereas *P. hypophthalmus* derives more than 95% of its oxygen uptake across the very large gills (Phuong et al., 2017b) in the same conditions (Lefevre et al., 2011a). This probably explains why *C. ornata* has a higher  $P_{aCO_2}$  (7 mmHg, at 27°C; Fig. 1C) than *P. hypophthalmus* (3 mmHg) at the same temperature (Damsgaard et al., 2018). *Chitala ornata* modulates air breathing, but not branchial ventilation, in response to changes in  $P_{aCO_2}$  (Tuong et al., 2019), and the persistence of this ventilatory response following branchial denervation indicates internal orientation of the sensors (Tuong et al., 2019). While in a water-breathing fish, the required elevation in  $P_{aCO_2}$  with elevated temperature from its low starting level requires a severe depression of ventilation relative to metabolism and hence reduced oxygen uptake (see Soncini and Glass, 1997), a bimodal breather can achieve the necessary increase in  $P_{aCO_2}$  without compromising oxygen uptake by simply changing its partitioning towards the air phase. Interestingly, this is exactly what is observed

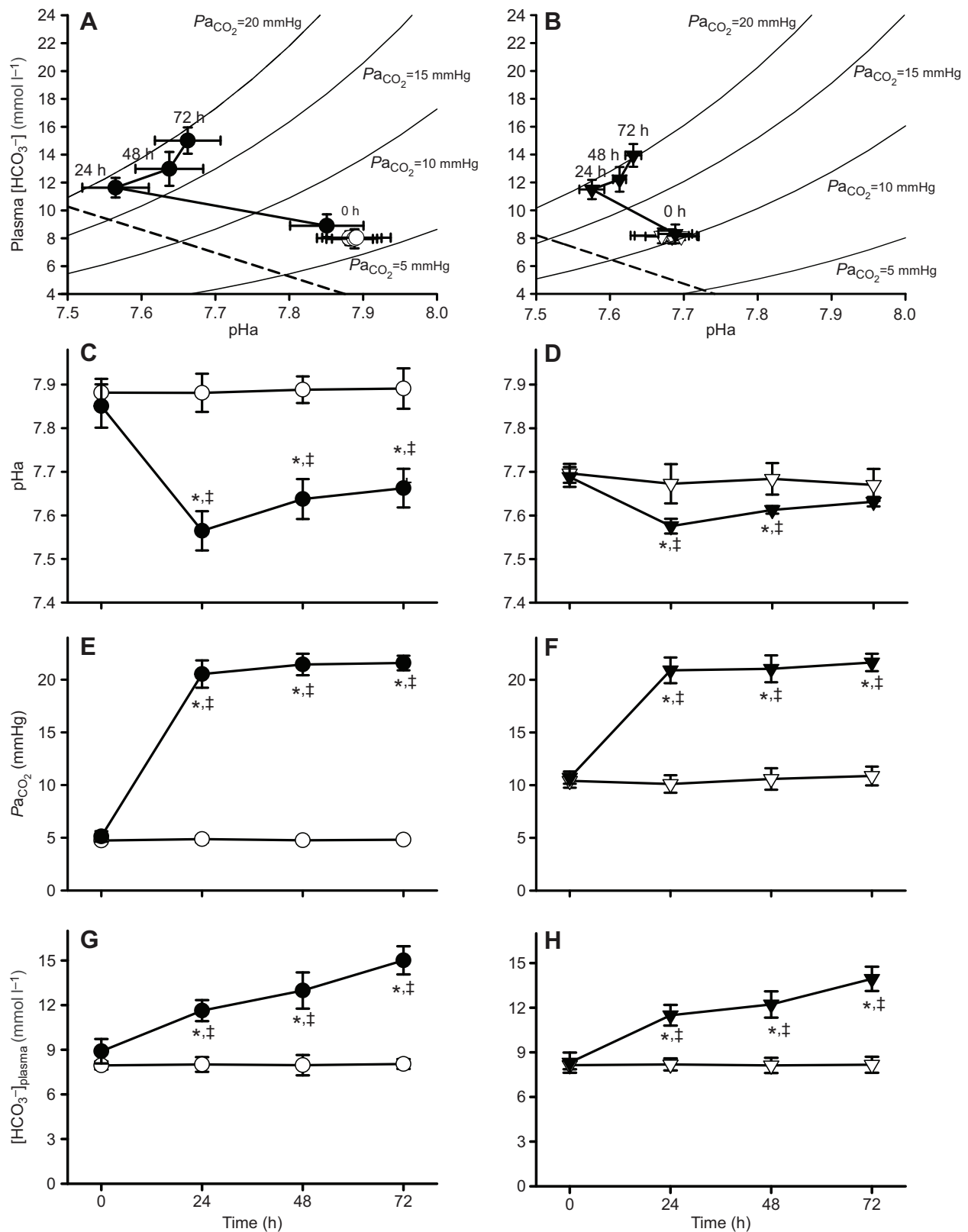


**Fig. 2. Effects of elevated temperature on gill ventilation and air-breathing frequency.** (A) Air-breathing frequency and (B) gill ventilation frequency after 1 h of exposure to elevated temperatures: 24, 27, 30, 33 and 36°C. The insets show representative traces of ventilation using impedance electrodes. A one-way ANOVA was used to compare temperatures. Different letters indicated significant differences ( $P < 0.05$ ). Data are means  $\pm$  s.e.m. ( $N = 6$ ).

in two species of gar (Rahn et al., 1971; Smatresk and Cameron, 1982), and in toads, which show a similar change in the partitioning from cutaneous to pulmonary gas exchange as temperature increases, with a similar attendant rise in  $P_{aCO_2}$  (Wang et al., 1998). We therefore interpret both the temperature-associated elevation in  $P_{aCO_2}$  and the accompanying change in ventilatory frequency in the present study as reflecting altered gas exchange partitioning for both oxygen and  $CO_2$ .

The exposure to 21 mmHg of aquatic hypercapnia at 25 and 33°C was designed to confirm the hypothesis that *C. ornata* defends the correct temperature-dependent pH<sub>a</sub> set point. To this end, the experiment was only partially successful. Hypercapnia enforced a respiratory acidosis at both temperatures (25 and 33°C; Fig. 3), and the resulting pH<sub>a</sub> disturbance measured at 24 h was greater at 25 than at 33°C (Fig. 3C,D), reflecting the lower initial  $P_{aCO_2}$  at the lower temperature (Fig. 3E,F). The recovery of pH<sub>a</sub> was more complete after 72 h at 33°C (not significantly different from controls) than at 25°C (Fig. 3A–D). Further, the regulatory rise in  $[HCO_3^-]$  at the two temperatures revealed similar responses (Fig. 3G,H), and did not appear to be slowed at the termination of





**Fig. 3. Effects of hypercapnia on pH<sub>a</sub> regulation at 25°C (left) and 33°C (right).** (A,B) Davenport diagrams with  $\text{CO}_2$  isopleths upon exposure to 21 mmHg [data for the non-bicarbonate buffer lines were taken from Gam et al., (2018) with temperature-dependent  $\text{pK}$  values, at the times indicated on the graph]. (C–F) The same data for pH<sub>a</sub>,  $\text{PaCO}_2$  and plasma  $\text{HCO}_3^-$  at each sampling time. Open symbols indicate normocapnic control series, filled symbols indicate 21 mmHg  $\text{CO}_2$  exposure group. A two-way ANOVA tested the difference from 0 h within treatment (asterisks) and between hypercapnic group and controls (double-dagger). Data are means  $\pm$  s.e.m. ( $N=6$ ).

the experiment. At 72 h, the increase in plasma  $[\text{HCO}_3^-]$  was accompanied by a greater than expected decrease in plasma  $[\text{Cl}^-]$ , and an additional drop in plasma  $[\text{Na}^+]$  and osmolality (Table S3). The change in  $[\text{Na}^+]$  is opposite to that expected from  $\text{Na}^+/\text{H}^+$  exchange, but probably reflects the osmolality change. Osmolality was reduced by ca. 10 mOsm, indicating a drop of ca. 5 mmol  $\text{l}^{-1}$  for both  $[\text{Na}^+]$  and  $[\text{Cl}^-]$ .  $[\text{Cl}^-]$  drops by 10 mmol  $\text{l}^{-1}$ , half of which is explained by  $\text{Cl}^-/\text{HCO}_3^-$  exchange and the other half by the change in osmolality. A decrease in osmolality with aquatic hypercapnia has been observed previously in this species (Gam et al., 2018) but presently remains unexplained. The high temperature group requires less  $\text{HCO}_3^-$  for full pH compensation than the low temperature group, and in our view more time would probably increase  $[\text{HCO}_3^-]$  and pH compensation further. A slow pH compensation is seen in other freshwater fish, and the tench (*Tinca tinca*) takes up to 2 weeks to elevate plasma  $\text{HCO}_3^-$  to the almost 40 mmol  $\text{l}^{-1}$  required for complete pH compensation at 10 mmHg  $\text{CO}_2$  (Jensen et al., 1993). Furthermore, the water concentrations of calcium, bicarbonate and chloride are important in determining both the speed and completeness of acid–base regulation during hypercapnia in fish (Larsen and Jensen, 1997). In the present case, the water levels of these ions were moderate [e.g. water  $[\text{Cl}^-] \sim 0.3 \text{ mmol l}^{-1}$  (Lefevre et al., 2011b);  $[\text{Na}^+] \sim 236 \pm 12 \mu\text{mol l}^{-1}$ ,  $[\text{K}^+] \sim 34 \pm 1$ ; and  $[\text{Ca}^{2+}] \sim 342 \pm 3 \mu\text{mol l}^{-1}$  (Yanagitsuru et al., 2019)], pointing to a somewhat delayed acid–base regulation.

In summary, we have demonstrated that this facultative air-breathing fish, which at low temperatures shows a  $P_{\text{aCO}_2}$  level only slightly elevated above that of water breathers, reduces pHa with increasing temperature by elevating  $P_{\text{aCO}_2}$  under constant  $[\text{HCO}_3^-]$ . We argue that this is a reflection of a regulated shift in the partitioning of gas exchange, which is probably common among bimodal breathers. Bayley et al. (2019) proposed that the elevated  $P_{\text{aCO}_2}$  is a pre-requisite for the utility of respiratory pH regulation because it radically reduces the problem of hypoventilation forming a constraint on oxygen uptake. These findings suggest that air breathing, and hence the partitioning of gas exchange, is to some extent regulated by acid–base status in air-breathing fish and suggest that these bimodal breathers will be increasingly likely to adopt respiratory pH control as temperature increases: an interesting avenue for future research.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: L.T.H.G., F.B.J., T.W., M.B.; Methodology: L.T.H.G., T.W., M.B.; Software: M.B.; Validation: M.B.; Formal analysis: L.T.H.G., F.B.J., T.W., M.B.; Investigation: L.T.H.G., D.T.T.H., D.D.T.; Resources: L.T.H.G., D.T.T.H., N.T.P., M.B.; Data curation: L.T.H.G., D.T.T.H., D.D.T.; Writing – original draft: L.T.H.G., D.T.T.H., F.B.J., M.B.; Writing – review & editing: L.T.H.G., F.B.J., T.W., M.B.; Visualization: L.T.H.G., D.D.T., M.B.; Supervision: D.T.T.H., M.B.; Project administration: D.T.T.H., N.T.P., M.B.; Funding acquisition: D.T.T.H., N.T.P., M.B.

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### Supplementary information

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