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ORIGINAL PAPER

Air-breathing changes the pattern for temperature-induced pH regulation in a bimodal breathing teleost

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Abstract It is well established that ectothermic vertebrates regulate a lower arterial pH when temperature increases. Typically, water-breathers reduce arterial pH by altering plasma [HCO_3^-], whilst air-breathers rely on ventilatory adjustments to modulate arterial PCO_2. However, no studies have investigated whether the shift from water- to air-breathing within a species changes the mechanisms for temperature-induced pH regulation. Here, we used the striped catfish *Pangasianodon hypophthalmus* to examine how pH regulation is affected by water- versus air-breathing, since *P. hypophthalmus* can accommodate all gas exchange by its well-developed gills in normoxic water, but achieves the same metabolic rate with aerial oxygen uptake using its the swim-bladder when exposed to aquatic hypoxia. We, therefore, measured arterial acid–base status in *P. hypophthalmus* as temperature changed between 20 and 35 °C in either normoxic or severely hypoxic water. In normoxic water, where *P. hypophthalmus* relied entirely on branchial gas exchange, *P. hypophthalmus* exhibited the typical teleost reduction in plasma [HCO_3^-] and arterial pH when temperature rose. However, when forced to increase air-breathing in hypoxic water, arterial PCO_2 fell due to a branchial hyperventilation, but it increased with temperature most likely due to passive CO_2 retention. We propose that the rise in arterial PCO_2 reflects a passive consequence of the progressive transition to air breathing at higher temperatures, and that this response fortuitously matches the new regulated pH_a, relieving the requirement for branchial ion exchange.

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Keywords Acid/base regulation · Blood gases · Carbon dioxide · Oxygen · Pangasius.

Introduction

Virtually all ectothermic animals reduce arterial pH (pH_a) when temperature increases (Austin et al. 1927; Rahn et al. 1971; Randall and Cameron 1973; Wood et al. 1978; Smatresk and Cameron 1982; Cameron and Kormanik 1982; Boutilier et al. 1987; Amin-Naves et al. 2004; Truchot 2012; Fobian et al. 2014). The regulated variable underlying this ubiquitous physiological pattern, be it ionization of the α-imidazole-groups on histidines or the relative alkalinity of water, remains as disputed as it is unresolved (Austin et al. 1927; Reeves 1972; Hazel et al. 1978; Heisler 1980; Wang and Jackson 2016). Nevertheless, given the omnipresence of the pH_a fall with temperature, there seems to be general consensus that the reduction in pH_a with increased temperature ($\Delta pH_a/\Delta T$) is a tightly regulated response, although direct evidence is limited.

The temperature-mediated pH_a reduction can be achieved by either transepithelial exchange of acid–base relevant ions or respiratory modulation of arterial PCO_2 (P_aCO_2) by altering ventilation relative to metabolic CO_2 production (i.e., air convection requirement) (Wang and Jackson 2016). The importance of these two distinct mechanisms differs between water and air breathers. Thus, due to the high ventilation rates imposed by oxygen's low water solubility, water-breathing vertebrates are typically obliged to alter plasma [HCO_3^-] ($[HCO_3^-]_{pl}$) by transepithelial ion exchange. In contrast, air breathers normally regulate pH_a by altering ventilation rates to change P_aCO_2.

Bimodal breathers, such as air-breathing fishes and many amphibians, also reduce pH_a as temperature

increases (Johansen 1970; Smatresk and Cameron 1982; Graham 1997; Amin-Naves et al. 2004; Wright and Turko 2016), but the underlying mechanisms are likely to differ according to the plethora of respiratory structures and species-specific dependence on aerial respiration. In this context, the air-breathing catfish *Pangasianodon hypophthalmus* represents an appropriate model to examine how pH_a regulation is affected by water versus air-breathing. This species tolerates temperatures over a wide range from 20 to 39 °C and is in possession of both large gills and well-vascularized air-breathing organ (Phuong et al. 2017). At temperatures between 27 and 39 °C, *P. hypophthalmus* can accommodate all gas exchange by its well-developed gills in normoxic water (Andersen et al. 2015), but resorts to air-breathing by virtue of its modified swim-bladder when exposed to aquatic hypoxia (Lefevre et al. 2011). This response is different compared to other bimodal breathing fishes that typically increase air-breathing frequency with temperature in normoxia.

We hypothesized that *P. hypophthalmus* regulates pH_a via active ion-exchange only (i.e., P_aCO_2 is constant and $[HCO_3^-]_{pl}$ is reduced) when exposed to increases in temperature in normoxic water where *P. hypophthalmus* can accommodate gas exchange entirely by water-breathing. In contrast, we hypothesized that the transition to air-breathing in hypoxic water would lead to pH_a-regulation being of respiratory origin (i.e., P_aCO_2 increases and $[HCO_3^-]_{pl}$ remains constant), as P_aCO_2 would increase passively due to an increased resistance in CO_2 unloading associated with air-breathing. Thus, by disentangling the effects of air-breathing, we also address the more general question as to whether the reduction in pH_a with increased temperature is indeed maintained during both air and water-breathing; If pH_a and $\Delta pH_a/\Delta T$ are indeed similar during both conditions, it would provide strong evidence for pH_a actually being the sensed variable, and that new set-point is regulated when body temperature changes.

Materials and methods

Animals

Juvenile *Pangasianodon hypophthalmus* were obtained from a local supplier (Credofish, Denmark) and raised at Aarhus University at 27 °C for at least 6 months in 1 m^3 tanks supplied with oxygenated water from a recirculating system. The fishes were fed to satiety with commercial dry pellets every day. All procedures described below were in accordance with the Danish rules for animal experimentation (2016-15-0201-00865).

Animal handling and experimental protocol

Pangasianodon hypophthalmus ($n = 20$, mass = 1590 ± 93 g, length = 48 ± 1 cm; means ± SEM) were anaesthetized in 0.15 g l^{-1} benzocaine. When unresponsive to handling, they were transferred to a surgical table where the gills were irrigated with well-oxygenated water containing 0.08 g l^{-1} benzocaine (Phuong et al. 2016). A polyethylene catheter (PE50) was inserted into the dorsal aorta (Soivio et al. 1975) and another catheter (PE50) was placed in the opercular cavity through the operculum. The fish were allowed to recover for 24 h, which is sufficient for complete acid/base recovery in *P. hypophthalmus* (Phuong et al. 2016), in individual 100 l normoxic holding tanks at 35 °C supplied with water from a central 500 l tank in which water PO_2 (P_wO_2) and temperature were regulated. This setup allowed fast equilibration of both temperature and P_wO_2 in the individual holding tanks, while the air-phase above water in those tanks remained normoxic. On the following day, ventilatory parameters were monitored for 30 min in each fish, whereupon a blood sample was withdrawn. Temperature was then decreased by 5 °C on each of the following days, and ventilation and blood parameters were measured at each temperature. Temperature was changed by ~2.5 °C h^{-1}, allowing ~22 h for compensation to the temperature reduction, which is sufficient for pH_a regulation in other air-breathing fishes (Smatresk and Cameron 1982). This protocol was completed in both normoxia (P_wO_2 > 130 mmHg, $n = 13$) and hypoxia (P_wO_2 = 25 mmHg, $n = 7$). The critical oxygen tension of *P. hypophthalmus* is 57 mmHg at 27 °C (Lefevre et al. 2011), and the hypoxic P_wO_2 level was chosen to represent 50% of the P_{crit} value, where the species is expected to use air-breathing to provide for almost all of its metabolic demand.

To remove a possible influence of the direction of temperature change (i.e., heating from 20 to 35 °C versus cooling from 35 to 20 °C) on our data interpretation, six of the normoxic fish were recovered at 20 °C, and temperature was then increased rather than decreased on the following days. We did not observe any effect of direction of temperature change on pH_a (a proxy for post-anesthesia acid/base recovery) all hypoxic fishes were cooled from 35 to 20 °C. This allowed us to calculate a Ventilation Index (VI) relative to 35 °C normoxia on day 1 (see calculation for VI below), where the hypoxic fish recovered from anesthesia in normoxia at 35 °C and ventilatory parameters were measured on the first day, after which the water was bubbled with pure N_2 in the central tank to obtain aquatic hypoxia.

Water oxygen level and temperature were monitored and regulated through a controller box (Respirometer, v. 1.5.0.c Aarhus University, Denmark) coupled with an optical O_2 probe (VisiFerm DO, Hamilton Process Analytics, NV, USA).

Blood analysis

Immediately after blood sampling, pH_a and P_aO_2 were measured on a GEM Premier 3500 automated blood gas analyzer (Instrumentation Laboratory, Bedford MA) using previously validated temperature compensation algorithms (Malte et al. 2014). Blood hemoglobin concentration was measured spectrophotometrically after conversion to cyanomethemoglobin using Drabkin's reagent. Plasma concentration of CO_2 ($[CO_2]_{pl}$) and blood concentration of O_2 ($[O_2]_{bl}$) were measured in duplicate using the methods described by Cameron (1971) and Tucker (1967), respectively. The remaining blood was centrifuged at 4000*g* for 3 min and the separated plasma stored at −80 °C for subsequent measurements of plasma Cl^- concentration ($[Cl^-]_{pl}$) using a chloride titrator (Sherwood model 926S MK II Chloride analyzer, Sherwood Scientific Ltd., Cambridge, UK). P_aCO_2 was calculated by rearranging the Henderson–Hasselbalch equation (Damsgaard et al. 2014) using p*K´* from (Boutilier et al. 1984) and α_{CO2} from (Dejours 1981). $[HCO_3^-]_{pl}$ was calculated by subtracting dissolved CO_2 from $[CO_2]_{pl}$ ($[HCO_3^-]_{pl}=[CO_2]_{pl} - \alpha_{CO2}\cdot P_aCO_2$). The concentration of hemoglobin-bound O_2 ($[HbO_2\text{-sat}]$) was calculated by subtracting plasma-dissolved O_2 from $[O_2]_{bl}$ ($[O_2]_{bl} - \alpha_{O2}\cdot P_aO_2$) using P_aO_2 from GEM and α_{O2} from (Dejours 1981). Fractional arterial saturation of hemoglobin with O_2 (HbO_2-sat) was calculated as the concentration of hemoglobin-bound O_2 relative to blood hemoglobin concentration. The measured P_aO_2 using the GEM 3500 is overestimated by up to 25%, but due to the low α_{O2} compared to $[O_2]_{bl}$, such errors will overestimate HbO_2-sat by less than 0.33% (Malte et al. 2014). For the same reason, P_aO_2 was calculated at all four temperatures from the HbO_2-sat and the Hill equation ($HbO_2\text{-sat} = {P_aO_2}^{n50}/({P_aO_2}^{n50} + {P_{50}}^{n50})$; P_{50}, partial pressure of oxygen at half saturation; n_{50}, Hill's cooperativity constant) using data from whole blood O_2 equilibrium curves (Damsgaard et al. 2015b), and these calculated P_aO_2 values are similar to the values determined with a Radiometer PO_2-electrode in a previous study (Damsgaard et al. 2015b). The values used for this model were $n_{50} = 1.5$, and $P_{50} = 1.3$, 3.2, 8.3, and 20.1 mmHg at 20, 25, 30, and 35 °C, respectively, and the same P_{50} was used for both the normoxic and hypoxic groups as the typical hypoxia-induced reduction in P_{50} due to lowered intra-erythroid [ATP] is not expected in *P. hypophthalmus* with ATP irresponsive Hb (Damsgaard et al. 2015b).

Measurements of cardio-respiratory parameters

The opercular and dorsal aortic catheters were connected to pressure transducers (PX600, Irvine, CA, USA) and pressure data were collected with a MP100 BIOPAC system (Biopac Systems Inc., CA, USA) at 200 Hz. The pressure transducers were two-point calibrated against a static water column on a daily basis with pressures similar to in vivo values. The fish tanks were covered with dark plastic to avoid visual disturbances during the experiment. Air-breathing frequency (f_{AB}) was determined from the characteristic changes in the pressure profile from the opercular catheter. This AB-signal was determined after observation of several ABs in different individuals prior to the experimental protocol and were easily distinguishable from normal ventilation. Movement of the fish did produce some noise on the signal, but only rarely did this interfere with the analysis. Total gill ventilation was expressed as a 30 min average for each fish at the given temperature, and expressed as the product of frequency (f_V) and amplitude (V_{AMP}). VI was calculated for each temperature (n), relative to 35 °C in normoxia:

$$\mathrm{VI}_{\mathrm{temp}(n)} = \frac{(\text{amplitude} \times \text{frequency})_{\mathrm{temp}(n)}}{(\text{amplitude} \times \text{frequency})_{\mathrm{temp}(35^\circ\mathrm{C})}} \times 100\% \quad (1)$$

Calculations and statistics

The temperature coefficient, Q_{10}, for a biological process (R) between two temperatures T_1 and T_2 was calculated as:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10^\circ\mathrm{C}}{T_2 - T_1}} \quad (2)$$

The effects of temperature and hypoxia were assessed by a mixed model analysis of variance using individual fish as random effect, temperature as repeated factor and hypoxia as treatment factor in R software (R Core Team, 2015, v. 3.2.2). Output from this analysis is tabulated in Table 1. A pairwise comparison test between normoxia and hypoxia at each temperature was performed, and P-values were adjusted using a Benjamini-Hochberg correction in InVivoStat software (InVivoStat v. 3.6.0). Data were inspected for homoscedasticity using a residual plot, and when heteroscedastic, data was rank transformed. Normality of all data was assessed from a normal probability plot. Significance level was set to 0.05.

Results

Acid/base regulation and oxygen saturation

In normoxic water, pH_a decreased with an average gradient of -0.0086 ± 0.0018 U $^oC^{-1}$ ($P < 0.001$; Fig. 2a) between 20 and 35 °C. However, this pH_a change appears non-linear and the change between 20 and 25 °C was not significant ($P = 0.45$). Between 25 and 35 °C, the pH_a decrease was linear with a slope of -0.013 ± 0.002 U $^oC^{-1}$

Table 1 Output of the mixed model ANOVA showing the effect of temperature and hypoxia on respiratory parameters

Parameter	All data	Effect of temperature		Effect of hypoxia
		Normoxia	Hypoxia	
pH_a	− 0.010 ± 0.0014***	− 0.0086 ± 0.0018***	− 0.013 ± 0.0012***	− 0.021 ± 0.017
P_aCO_2 [mmHg]	0.048 ± 0.017**	0.0096 ± 0.64	0.13 ± 0.011***	− 0.94 ± 0.22***
$[HCO_3^-]_{pl}$ [mM]	− 0.055 ± 0.0614	− 0.17 ± 0.080*	0.20 ± 0.019***	− 3.8 ± 0.80***
HbO_2-sat [%]	− 0.56 ± 0.39	− 0.28 ± 0.50	− 1.1 ± 0.52*	− 46 ± 5.2***
Hct [%]	− 0.012 ± 0.13	− 0.092 ± 0.17	0.16 ± 0.14	− 0.61 ± 3.5
[Hb] [mM]	0.0042 ± 0.022	− 0.003 ± 0.03	0.021 ± 0.020	− 0.11 ± 0.78
MCMC [mM]	− 0.038 ± 0.064	− 0.024 ± 0.069	− 0.072 ± 0.14	0.38 ± 0.81
$[Cl^-]_{pl}$ [mM]	0.50 ± 0.11***	0.69 ± 0.13***	0.0061 ± 0.20	4.8 ± 2.8
f_{AB} [hr^{-1}]	0.45 ± 0.11***	− 0.032 ± 0.048	1.4 ± 0.15***	9.5 ± 1.3***
f_V [min^{-1}]	1.2 ± 0.26***	1.2 ± 0.38**	1.1 ± 0.24***	5.9 ± 5.1
Rel. V_{AMP} [%]	0.88 ± 0.94	1.4 ± 1.2	0.13 ± 1.6	61 ± 11***
VI [%]	1.9 ± 1.1	1.8 ± 1.4	1.9 ± 1.8	56 ± 13**
MAP [cmH_2O]	0.33 ± 0.054***	0.35 ± 0.054***	0.28 ± 0.13*	− 1.4 ± 1.3
f_H [min^{-1}]	2.7 ± 0.39***	2.6 ± 0.5***	3.0 ± 0.64***	− 5.7 ± 6.7

Values are given as the estimate effect ± standard error. *, **, and *** indicate P values of < 0.05, < 0.01, and < 0.001, respectively. See main text for abbreviations

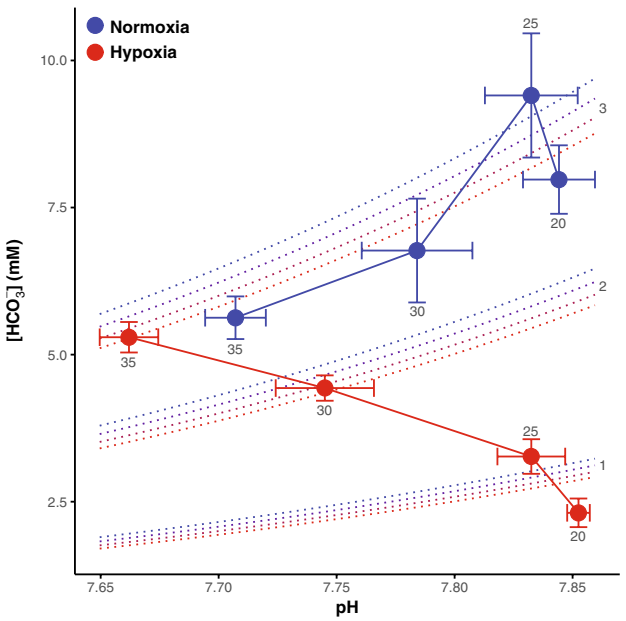

Fig. 1 Davenport diagram showing extracellular acid–base regulation in cannulated *Pangasianodon hypophthalmus* in response to 5 °C-temperature changes between 20 and 35 °C during aquatic normoxia (P_wO_2 > 130 mmHg; blue-lined symbols) and aquatic hypoxia (P_wO_2 = 25 mmHg; red-lined symbols). Isopleths are color coded for temperature (blue to red for increasing temperature). Isopleths were generated using pK' from (Boutilier et al. 1984) and α_{CO2} from (Dejours 1981). Data are means ± standard error of mean (n = 13 for normoxia, 7 for hypoxia). (Color figure online)

(Fig. 1). This was associated with a reduction in $[HCO_3^-]_{pl}$ (− 0.17 ± 0.080 mM $^oC^{-1}$; P < 0.05; Figs. 1, 2c) at constant P_aCO_2 (P = 0.64; Figs. 1, 2b). The fall in $[HCO_3^-]_{pl}$ was attended by an increase in $[Cl^-]_{pl}$ (0.50 ± 0.11 mM $^oC^{-1}$; P < 0.001, Table 2).

Arterial pH was not affected by water oxygen levels (P = 0.20; Fig. 2a). During hypoxia, P_aCO_2 rose approximately three-fold from 20 to 35 °C (0.13 ± 0.011 mmHg $^oC^{-1}$; P < 0.001; Figs. 1, 2b) attended by a rise in $[HCO_3^-]_{pl}$ of 3 mM (− 13.3 ± 1.9 slykes (P < 0.001); 0.20 ± 0.019 mM $^oC^{-1}$ (P < 0.001); Figs. 1, 2c). Arterial HbO_2-sat was higher during normoxia (P < 0.001) and decreased with temperature during hypoxia (P < 0.001), but not in normoxia (P = 0.87; Fig. 3).

Cardioventilatory responses to temperature in normoxic and hypoxic water

In normoxic water, the ventilation index increased [however, not significantly (P = 0.20)] with a Q_{10} of 1.4 through a significant elevation in f_V as temperature rose (1.2 ± 0.38 min^{-1} $°C^{-1}$ (P < 0.001)), while there were no changes in rel. V_{AMP} (P = 0.23) or f_{AB} (P = 0.49) (Fig. 4). Gill ventilation frequency was unaffected by hypoxia (P = 0.23), but rel. V_{AMP} and f_{AB} were higher in hypoxia (both P < 0.001) (Fig. 4), resulting in an overall higher VI in hypoxia (P < 0.01), that, however, was only significantly different at 25 °C (P < 0.01). As in hypoxia, increases in temperature resulted in higher f_V (1.1 ± 0.24 min^{-1} $°C^{-1}$ (P < 0.001)), but not in Rel. V_{AMP} (P = 0.25) and VI (P = 0.53, Q_{10} = 0.88). Air-breathing was absent at 20 °C during normoxia and hypoxia, but f_{AB} increased with temperature during hypoxia (1.4 ± 0.15 h^{-1} $°C^{-1}$ (P < 0.001)), resulting in higher f_{AB} at 25, 30, and 35 °C (P = 0.025, < 0.001, and < 0.001, respectively) (Fig. 4a).

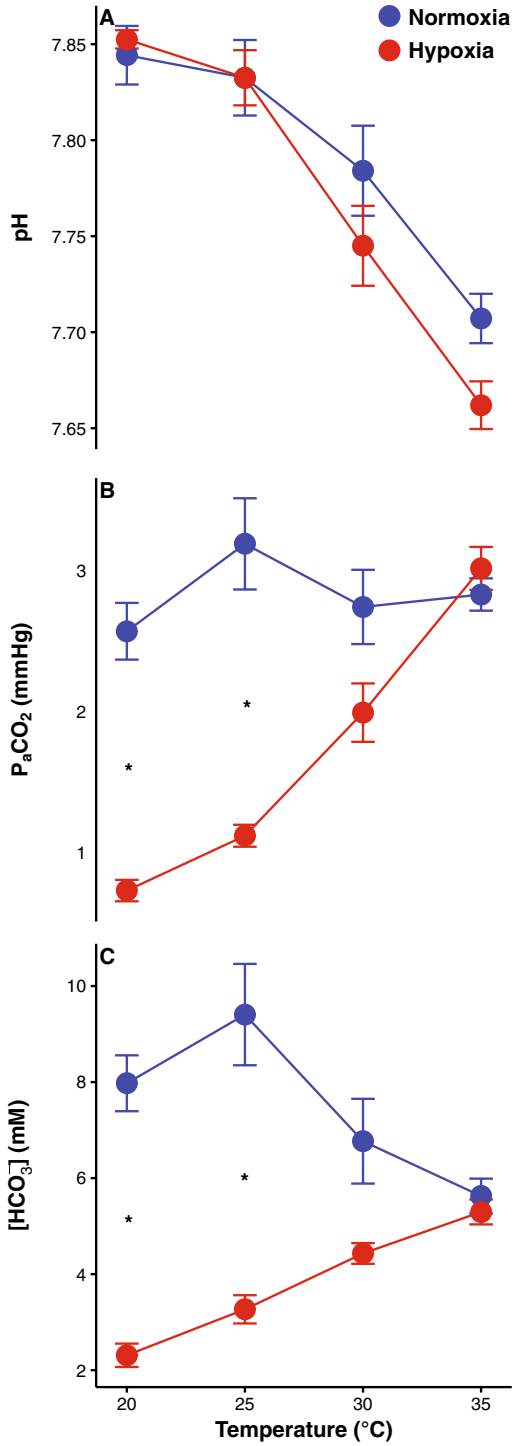

Fig. 2 Extracellular acid–base regulation in cannulated *Pangasianodon hypophthalmus* in response to temperature changes between 20 and 35 °C during aquatic normoxia (P_wO_2 > 130 mmHg; blue symbols) and aquatic hypoxia (P_wO_2 = 25 mmHg; red symbols). **a** arterial pH (pH_a), **b** arterial PCO_2 (P_aCO_2), and **c** plasma bicarbonate concentration ($[HCO_3^-]_{pl}$). Asterisks indicate significant difference in a parameter between the normoxic and hypoxic groups at a specific temperature. Data are means ± standard error of mean (n = 13 for normoxia, 7 for hypoxia). (Color figure online)

Both f_H and MAP increased with temperature (2.7 ± 0.4 min^{-1} $°C^{-1}$ ($P < 0.001$), and 0.33 ± 0.05 cmH_2O $°C^{-1}$ ($P < 0.001$), respectively), and were unaffected by aquatic hypoxia ($P = 0.65$, and 0.31, respectively) (Fig. 5).

Discussion

The reduction in pH_a as temperature rose ($\Delta pH_a/\Delta T$) in *P. hypophthalmus*, resembles the archetypical fall of ectothermic vertebrates (Rahn et al. 1971; Randall and Cameron 1973; Wood et al. 1978; Smatresk and Cameron 1982; Cameron and Kormanik 1982; Boutilier et al. 1987; Amin-Naves et al. 2004; Fobian et al. 2014), and pH_a and $\Delta pH_a/\Delta T$ did not depend on whether *P. hypophthalmus* was exclusively water-breathing in normoxic water or bimodal-breathing in hypoxic water, where air-breathing frequencies approached the high range observed in other air-breathing fishes (Graham 1997). However, the acid–base status was strikingly different. Thus, pH_a fell exclusively through a reduction in $[HCO_3^-]_{pl}$ in normoxia, whereas elevated P_aCO_2 accounted for almost all of the temperature associated pH_a change in hypoxic water. *P. hypophthalmus*, therefore, exhibits an intraspecific shift of the classic distinction between water- and air-breathers in the acid–base response to altered temperature. This shift bears resemblance to the transition in regulation between air- and water breathers within the sarcopterygian lineage and is normally ascribed to the emergence of central chemoreception for CO_2/pH in amniotes allowing for ventilatory regulation of P_aCO_2. There is, however, no indication of central chemoreception affecting ventilation in *P. hypophthalmus* (Thomsen et al. 2017), and we suggest that the rise in P_aCO_2 with elevated temperature in hypoxia is a passive response arising from the increased aerial respiration and temperature-independent branchial ventilation in hypoxia rather than a regulated process *per se*.

P. hypophthalmus relies almost exclusively on branchial gas exchange in normoxic water (Lefevre et al. 2011), and it is not surprising that it follows the typical piscine pattern of achieving the $\Delta pH_a/\Delta T$ by means of decreasing $[HCO_3^-]_{pl}$ (Randall and Cameron 1973; Heisler 1980). Given the reduction in $[Cl^-]_{pl}$, it seems that branchial HCO_3^-/Cl^- exchange mediates the reduction in $[HCO_3^-]_{pl}$ with increased temperature, even though it does not follow the classical equimolar exchange rate (Grosell et al. 2009), which may have been due to non-equimolar exchange rates, H^+/Na^+-exchange, and/or experimental error (Evans et al. 2005; Perry and Gilmour 2006; Damsgaard et al. 2015a; Hvas et al. 2016). The temperature independence of P_aCO_2 during normoxia was consistent with the matching of gill ventilation to the rise in standard metabolic rate (SMR), where Q_{10} for gill VI of 1.4 (25–35 °C) is similar to the Q_{10} of 1.6 for SMR [27–36 °C;

Table 2 Plasma concentrations of Na^+, K^+, and Cl^-, and osmolarity in Pangasianodon hypophthalmus during acclimation to 20–35 °C and normoxia or hypoxia

	20 °C		25 °C		30 °C		35 °C	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
$[Na^+]_{pl}$ [mM]	129 ± 1.55	133 ± 3.01	129 ± 2.83	137 ± 1.26	135 ± 2.08	138 ± 0.746	133 ± 1.09	134 ± 1.9
$[K^+]_{pl}$ [mM]	2.69 ± 0.236	2.45 ± 0.0423	2.67 ± 0.242	2.59 ± 0.0576	2.93 ± 0.114	2.67 ± 0.142	2.79 ± 0.0892	2.64 ± 0.145
$[Cl^-]_{pl}$ [mM]	100 ± 1.04	111 ± 1.77	99.8 ± 2.33	108 ± 0.917	107 ± 2.02	109 ± 0.748	112 ± 0.634	111 ± 3.27
Osm [mOsm]	246 ± 2.57	253 ± 4.41	247 ± 4.73	258 ± 1.32	259 ± 3.83	257 ± 2.03	264 ± 2.83	271 ± 5.36

Data are means ± standard error of mean ($n = 13$ for normoxia, 7 for hypoxia)

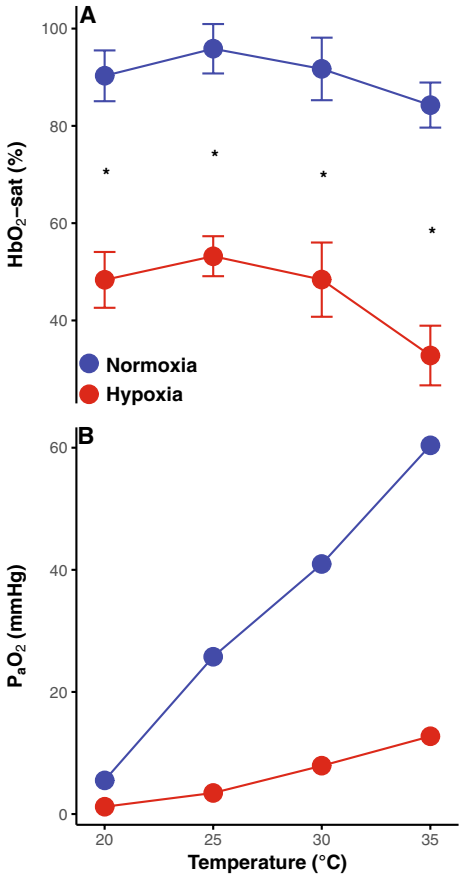

Fig. 3 **a** Arterial oxygen saturation (HbO_2-sat) and **b** calculated arterial PO_2 (P_aO_2) in cannulated *Pangasianodon hypophthalmus* in response to temperature changes between 20 and 35 °C during aquatic normoxia (P_wO_2 > 130 mmHg; blue-lined symbols) and aquatic hypoxia (P_wO_2 = 25 mmHg; red lined symbols). Asterisks indicate significant difference in a parameter between the normoxic and hypoxic groups at a specific temperature. Data are means ± standard error of mean ($n = 13$ for normoxia, 7 for hypoxia). (Color figure online)

(Andersen et al. 2015)]. In addition, there was no increase in air-breathing events with increased temperature in normoxic water. This pattern contrasts to the air-breathing gar and South American lungfish, where air-breathing frequency increases as temperature stimulates metabolism (Smatresk and Cameron 1982; Amin-Naves et al. 2004), but resembles the air-breathing Alaska blackfish (Lefevre et al. 2014).

In hypoxia, *P. hypophthalmus* exhibited a marked branchial hyperventilation (significant at 25 °C), which is atypical for a bimodal breather with high capacity for aerial oxygen uptake. The increased gill ventilation in hypoxia, where CO_2 is excreted at a higher rate compared to in normoxia, explains the lower PCO_2 in hypoxia at the lower temperatures. Due to the higher capacitance coefficient for oxygen in air compared to water, a shift from water to air-breathing provides for decreased ventilation rates, leading to elevated PCO_2 in blood and tissues (Rahn 1966), and explains why P_aCO_2 increases in many facultative air-breathing fishes as they switch to aerial respiration in hypoxic water (Shartau and Brauner 2014; Wright and Turko 2016). In these cases, there is a typical reduction in branchial CO_2 excretion caused by reduced perfusion of the secondary branchial lamellae. We did not measure the partitioning of gas exchange in *P. hypophthalmus* in this study, but the rise in air-breathing with temperature indicates that the increase in P_aCO_2 with elevated temperatures in hypoxic water is a passive consequence of the progressive transition to aerial respiration that was enforced by the increased metabolism and associated CO_2 production with increased temperatures. As such, *P. hypophthalmus* behaves akin to skin-breathing salamanders where increased P_aCO_2 causes a pH_a reduction with increased temperature through a fortuitous matching of the temperature effects on CO_2 production and the cutaneous conductance for CO_2 (Moalli et al. 1981). In fact, in *P. hypophthalmus*, it appears that the rise in P_aCO_2 with temperature elevation would have caused a larger fall in pH_a than predicted by normoxic pH_a regulation. *Pangasianodon hypophthalmus*, therefore, regulates pH_a by transepithelial ion exchange to elevate $[HCO_3^-]_{pl}$, which is a response also observed in other bimodal breathing fishes (Smatresk and Cameron 1982; Amin-Naves et al. 2004). This shows that despite of having markedly different P_aCO_2 in normoxia and hypoxia, *P. hypophthalmus* still regulate pH_a to achieve the same pH_a set-point, and hence the pH_a set-point is P_aCO_2 independent. This suggests that it is pH_a that is sensed by a

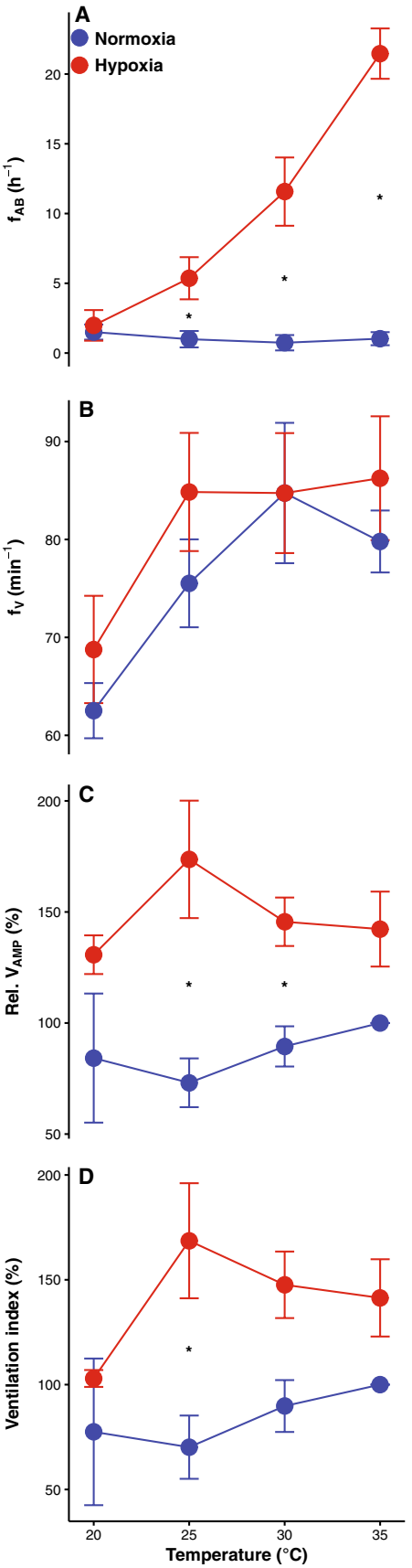

Fig. 4 Ventilatory regulation in cannulated *Pangasianodon hypophthalmus* in response to temperature between from 20 to 35 °C during aquatic normoxia (P_wO_2 > 130 mmHg; blue symbols) and aquatic hypoxia (P_wO_2 = 25 mmHg; red symbols). **a** Air-breathing frequency (f_{AB}). **b** Branchial ventilation frequency (f_V), **c** relative ventilation amplitude (rel. V_{AMP}), and **d** Branchial Ventilation Index (VI). Relative ventilation amplitude and Ventilation Index for individual fish were calculated relative to the value in normoxia at 35 °C. Asterisks indicate significant difference in a parameter between the normoxic and hypoxic groups at a specific temperature. Data are means ± standard error of mean (n = 13 for normoxia, 7 for hypoxia). (Color figure online)

hitherto unidentified pH_a sensor and that this sensor regulates pH_a by altering trans-epithelial ion-exchange rates, where positive deviations in set-point pH_a result in enhanced HCO_3^- excretion rates (and vice versa). The changes in pH_a with temperature follows the changes in pK of α-imidazole, and since pH_a might be the sensed parameter, this points to the α-imidazole on a surface-exposed histidine being the sensor of pH_a and the mediator of temperature-induced pH_a regulation.

Pangasianodon hypophthalmus had lower HbO_2-sat and P_aO_2 across all temperatures during aquatic hypoxia. Interestingly, P_aO_2 was lower than P_wO_2 at all temperatures showing that blood did not fully equilibrate to P_wO_2 during the branchial transit. Branchial oxygen loss can, therefore, not account for the arterial desaturation, which has been suggested to be a problem for air-breathing fishes during aquatic hypoxia (Randall 1981; Graham 1997; Scott et al. 2017). In contrast, it is plausible that perfusion of the secondary lamellae is highly reduced during hypoxia curtailing such branchial loss, and that the low oxygen levels in the dorsal aorta represents the mixture of oxygenated blood from the swim-bladder and systemic venous return that merge at the inflow to the heart. This would resemble the bowfin (*Amia calva*), which displays a similar 25% desaturation, and where flow measurements indicated this was caused by the mixing of fully saturated blood leaving the ABO and venous blood (Randall et al. 1981). Regardless of the cause of the low HbO_2-sat, branchial oxygen uptake could have been augmented through increases in pH_a to left-shift the hemoglobin O_2 binding curve. This illustrates the tradeoff between pH_a- and oxygen homeostasis at higher temperatures in bimodal- and water-breathing vertebrates, where pH_a regulation is prioritized over oxygen uptake at higher temperatures in *P. hypophthalmus* compromising blood–oxygen transport. A similar compromise between oxygen delivery and acid–base balance by virtue of lowering ventilation relative to metabolic CO_2 production at high temperature has also been proposed for amphibians and reptiles (e.g., Stinner 1987; Wang et al. 1998).

In conclusion, we demonstrate *P. hypophthalmus* exhibits intraspecific shift in the pattern for temperature-induced pH_a modulation, but that $\Delta pH_a/\Delta T$ and the actual

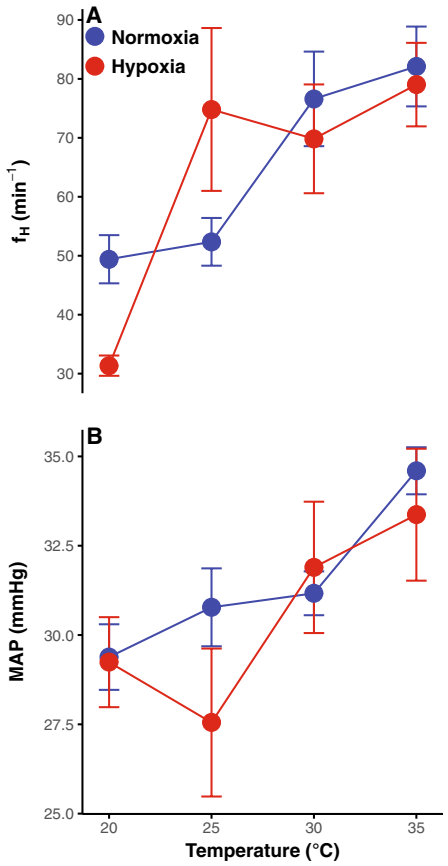

Fig. 5 Cardiovascular regulation in cannulated *Pangasianodon hypophthalmus* in response to temperature changes between 20 and 35 °C during aquatic normoxia (P_wO_2 > 130 mmHg; blue symbols) and aquatic hypoxia (P_wO_2 = 25 mmHg; red symbols). **a** heart rate (f_H) and **b** mean arterial blood pressure (MAP). Asterisks indicate significant difference in a parameter between the normoxic and hypoxic groups at a specific temperature. Data are means ± standard error of mean (n = 13 for normoxia, 7 for hypoxia). (Color figure online)

pH_a are independent whether the temperature-induced fall in pH_a is achieved by changes in $[HCO_3^-]_{pl}$ during water-breathing or via changes in P_aCO_2 during hypoxia. This was achieved without central chemoreception and limited the requirements for transepithelial ion-exchange. This finding provides strong evidence that pH_a is indeed regulated around a new set-point when temperature changes, and since the changes in pH_a follow the change in pK of α-imidazole, our data lend support to the idea of surface-exposed histidines sense pH_a and mediate the acid/base regulation as originally proposed in Reeves' alphastat hypothesis (Reeves 1972). Second, our study provides insight to the physiological tradeoffs at higher temperatures between interconnected physiological systems, such as O_2 transport and acid/base regulation. We emphasize the need to understand how such systems are limited and prioritized at higher temperatures to obtain a more holistic understanding of how such elevations are likely to affect organismal performance in a future warmer world.

Compliance with ethical standards

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Conflict of interest No competing interests declared.

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