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1 **High affinity and temperature sensitivity of blood oxygen binding in**
2 ***Pangasianodon hypophthalmus* due to lack of chloride-hemoglobin**
3 **allosteric interaction**

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10 **Running head:** Oxygen binding of pangasius blood and hemoglobin

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17 **ABSTRACT**

18 Air-breathing fishes represent interesting organisms in terms of understanding the physiological
19 changes associated with the terrestrialization of vertebrates, and are further of great socio-economic
20 importance for aquaculture in South East Asia. In order to understand how environmental factors
21 such as high temperature affect O₂ transport in air-breathing fishes, this study assessed the effects of
22 temperature on O₂ binding of blood and hemoglobin (Hb) in the economically important air-
23 breathing fish *Pangasianodon hypophthalmus*. To determine blood O₂ binding properties, blood
24 was drawn from resting cannulated fishes and O₂ binding curves made at 25 and 35°C. To
25 determine the allosteric regulation and thermodynamics of Hb O₂ binding, Hb was purified and O₂
26 equilibria recorded at 5 temperatures in the absence and presence of adenosine triphosphate (ATP)
27 and Cl⁻. Whole blood had a high O₂ affinity (O₂ tension at half saturation P_{50} = 4.6 mmHg at
28 extracellular pH 7.6 and 25°C), a high temperature sensitivity of O₂ binding (apparent heat of
29 oxygenation ΔH_{app} =-28.3 kcal mol⁻¹), and lacked a Root effect. Further, the data on Hb revealed
30 weak ATP binding and a complete lack of Cl⁻ binding to Hb, which in part explains the high O₂
31 affinity and high temperature sensitivity of blood O₂ binding. This study demonstrates how a potent
32 mechanism for increasing O₂ affinity is linked to increased temperature sensitivity of O₂ transport,
33 and provides a basic framework for a better understanding of how hypoxia-adapted species will
34 react to increasing temperatures.

35
36 **Keywords:** pangasius, oxygen affinity, ATP, chloride, allostery

GLOSSARY

39		
40	ATP	Adenosine triphosphate
41	GTP	Guanosine triphosphate
42	Hb	Hemoglobin
43	[Hb]	Blood tetrameric hemoglobin concentration
44	[HbO ₂]	Concentration of hemoglobin bound oxygen
45	Hct	Hematocrit
46	IEF	Isoelectrofocussing
47	MCHC	Mean corpuscular tetrameric hemoglobin concentration
48	n_{50}	Hill cooperativity coefficient at half saturation
49	NTP	Nucleotide triphosphate
50	[O ₂]	Blood oxygen concentration
51	P_{50}	Partial pressure of oxygen at half saturation
52	PCO_2	Partial pressure of carbon dioxide
53	pH _e	Extracellular pH
54	pH _i	Intracellular pH
55	pI	Isoelectric point
56	PO_2	Partial pressure of oxygen
57	R	Ideal gas constant
58	RBC	Red blood cell
59	S	Fractional oxygen saturation
60	α_{O_2}	Solubility coefficient of oxygen
61	β_{NHE}	β -adrenergically stimulated Na ⁺ /H ⁺ exchange
62	ΔH_{app}	Apparent enthalpy of oxygenation
63	ΔH^{cc}	Enthalpy of oxygenation-linked conformational changes
64	$\Delta H^{effector}$	Enthalpy of allosteric effector binding
65	ΔH^{H_2O}	Enthalpy of O ₂ solvation
66	ΔH^{O_2}	Intrinsic enthalpy of heme oxygen binding
67	ϕ	Bohr factor
68		

INTRODUCTION

The South East Asian striped catfish (*Pangasianodon hypophthalmus*) is an active, facultative air-breathing teleost with a modified and highly vascularized swim bladder enabling efficient aerial gas exchange (33, 34). The respiratory physiology of air-breathing fishes has been less studied than in water breathers and knowledge concerning the effects of temperature on O₂ uptake and transport in air-breathing fishes remains scarce. In addition, air-breathing fishes represent one of the fastest growing protein sources in the world (35) and *P. hypophthalmus* is of particular economic importance in South East Asian aquaculture. Therefore, it is of both academic and economic interest to gain insight into their respiratory physiology, and to understand the influence of key environmental factors, such as O₂ availability and temperature.

The optimal O₂ affinity of blood is a compromise between O₂ uptake from the environment and O₂ unloading at the tissues (6, 55). The O₂ content in air is much higher than in water (12), and blood O₂ saturation in air-breathers is therefore normally not considered limiting, which allows for a reduced O₂ affinity promoting efficient O₂ unloading at the tissues. However, fishes inhabiting hypoxic waters tend to have high blood O₂ affinity to secure O₂ uptake, despite unloading being potentially compromised. Air-breathing in fishes is believed to have evolved in response to ambient hypoxia (17), and the more ancestral air-breathing fishes with poorly developed air-breathing organs are therefore likely to have had high blood O₂ affinity to increase branchial O₂ uptake. However, as the air-breathing organs became more efficient, it gradually became possible to reduce blood O₂ affinity to facilitate unloading (10, 30). Other factors also act in opposing directions on blood O₂ affinity, including cardiovascular arrangement (29), O₂ carrying capacity (29, 42) and environmental O₂ availability (46), resulting in the continuum of blood O₂ affinities reported in air-breathing fishes.

Blood O₂ affinity is a function of the various expressed hemoglobin (Hb) isoforms combined intrinsic O₂ affinities, and their responses to red blood cell (RBC) allosteric effectors (63). Hb-O₂ affinity is modulated by temperature and by interactions with protons and CO₂ (facilitating O₂ unloading at the tissues via the Bohr effect), which aid unloading of O₂ in the tissues by stabilizing Hb in its low affinity tense (T) state conformation. In some fish Hbs, proton binding completely stabilizes the T state at low pH, resulting in loss of cooperative O₂ binding and incomplete O₂ saturation at high partial pressure of oxygen (*PO*₂) (Root effect) (24). This property enables O₂ off-loading to a very high *PO*₂ and hence swimbladder filling at depth and oxygenation of ocular and other tissues (4, 47, 48). Further, fish Hbs normally bind RBC Cl⁻ and adenosine

101 triphosphate (ATP) (44) providing allosteric regulation of O₂ binding (63). Hypoxia-induced
102 decreases in RBC [ATP] normally induce increased blood O₂ affinity (51, 68), while [Cl⁻] is
103 relatively constant in erythrocytes and tend to decrease Hb-O₂ affinity. Moreover, many fishes
104 possess a multitude of α - and β -globin genes (43), and their polypeptide products are incorporated
105 into functionally distinct Hb tetramers ($\alpha_2\beta_2$) (5, 13, 14, 45, 64), whose relative expression further
106 influences blood O₂ affinity.

107 Binding of O₂ to Hb is exothermic, hence Hb-O₂ affinity decreases with increasing
108 temperature. The apparent enthalpy of oxygenation (ΔH_{app}) is a sum of the intrinsic enthalpies of
109 heme O₂ binding (ΔH^{O_2}), of oxygenation-linked conformational changes (ΔH^{cc}), of O₂ solvation
110 (ΔH^{H_2O}) and of allosteric effector binding ($\Delta H^{effector}$) (62). ΔH^{O_2} and ΔH^{H_2O} are rather invariable
111 across species, hence the temperature sensitivity of O₂ binding is largely dependent on ΔH^{cc} and
112 oxygenation-linked endothermic release of allosteric effectors (61). Therefore, a relationship exists
113 between the number of binding sites for allosteric effectors and the temperature sensitivity of O₂
114 binding (8, 60, 62), where Hbs with multiple binding sites have a low temperature sensitivity, and
115 *vice versa*. It has been suggested that the higher number of effector binding sites, and thus low
116 temperature sensitivity of O₂ binding, is beneficial in regionally heterothermic animals, where O₂
117 unloading can for example, be safeguarded to the extremities of arctic mammals (7, 8, 15), and
118 where O₂ delivery can be regulated to warm brains and swimming muscles of tuna, shark and
119 billfishes (9, 31, 62). The thermodynamic consequences of reduced effector binding on Hb-O₂
120 affinity has received little attention in the literature but would analogously increase the temperature
121 sensitivity of Hb and blood O₂ binding.

122 To establish a better understanding of the effect of temperature on O₂ transport in the
123 air-breathing fish *P. hypophthalmus*, we measured the O₂ binding properties of blood and the effect
124 of temperature and pH, and then focused on describing the thermodynamics and allosteric
125 regulation of Hb O₂ binding by RBC allosteric effectors. Finally, we investigated how temperature
126 acclimation was associated with changes in the expression patterns of electrophoretically distinct
127 Hb isoforms.

128

129

MATERIALS AND METHODS

130 *Animal handling*

131 *Pangasianodon hypophthalmus* (Sauvage, 1878) were purchased from local aquaculture suppliers,
132 transferred to Can Tho University (Vietnam) and held for several months before experimentation.

133 The fishes were kept in large tanks with normoxic water at 27°C. The fishes were fed daily to
134 satiation with commercial purchased dry pellets and water was exchanged every third day. A
135 second group of fishes was acclimated under similar conditions but at 33°C to assess whether
136 temperature acclimation was associated with changes in the expression pattern of
137 electrophoretically distinct Hb isoforms.

138

139 *Surgical procedures*

140 14 fishes were anaesthetized by immersion in water containing 0.1 g benzocaine l⁻¹. When
141 immobile, the fishes were transferred to a surgical table where anesthesia was maintained by
142 flushing the gills with water containing 0.05 g benzocaine l⁻¹. A PE50 catheter was inserted into the
143 dorsal aorta through the roof of the mouth and extended through a hole in the rostrum (50). The
144 catheter was secured to the dorsal roof of the mouth and at the dorsal side of the fish by sutures and
145 the fish was allowed to recover for 24-72 h in normoxic water at 27°C, while the catheters were
146 flushed daily with heparinized saline. For blood measurements, a sample of up to 10 ml was taken
147 to determine arterial blood gas tensions and to construct blood O₂ equilibrium curves *in vitro*. All
148 experiments were performed in accordance with national guidelines for the protection of animal
149 welfare in Vietnam.

150

151 *Arterial blood parameters*

152 Arterial partial pressure of carbon dioxide (*PCO*₂) and extracellular pH (pH_e) were measured in
153 each fish using a GEM Premier 3500 automated blood gas analyzer (Instrumentation Laboratory,
154 Bedford, MA, USA) (40). At *PCO*₂ of 115 and 230 mmHg (16 and 32%), pH_e was extrapolated
155 from a log*PCO*₂ vs. pH_e plot due to the inability of the blood analyzer to measure pH_e below 6.8.
156 Arterial *PO*₂ was measured in 4 fishes using a Radiometer oxygen electrode thermostatted at 27°C
157 and connected to a PHM 71 (Radiometer, Copenhagen, Denmark). Hematocrit (Hct) was found as
158 the fractional RBC volume after centrifugation at 12,000 rpm for 3 min, and blood tetrameric Hb
159 concentration ([Hb]) was determined spectrophotometrically after conversion to cyanometHb using
160 Drabkin's reagent. Erythrocyte nucleoside triphosphates ([NTP]=[ATP]+[GTP]) were measured
161 spectrophotometrically (Cecil CE2041, Cambridge, UK) via enzyme-coupled reactions (Sigma
162 Bulletin no. 366-UV), using neutralized supernatants from blood deproteinized in 12%
163 trichloroacetic acid. Whole blood [NTP] was converted to erythrocyte [NTP] via the corresponding
164 Hct.

165

166 *Blood Tonometry for determination of whole blood oxygen binding properties*

167 Freshly drawn blood was divided into two Eschweiler (Kiel, Germany) tonometers and equilibrated
168 with humidified gas mixtures delivered from serially linked Wösthoff (Bochum, Germany) gas
169 mixing pumps. For blood O₂ equilibrium curves each tonometer was equilibrated with 3.8 or 22.8
170 mmHg CO₂ (0.5 or 3%) at either 25 or 35°C. Blood was equilibrated with 30% O₂ to determine the
171 O₂ carrying capacity, whereupon PO₂ was lowered to achieve O₂ saturations between 10 and 90%.
172 At each equilibration step the blood was allowed to equilibrate with the gas for ~ 30 min and blood
173 [O₂] was measured in duplicate with the Tucker method (52). To quantify the Root effect, blood O₂
174 saturation was measured at 25°C while equilibrated with air during progressive increases in PCO₂
175 from 3.8 to 243 mmHg (0.5% to 32%). In some fish with a smaller blood volume, it was only
176 possible to perform one O₂ equilibrium curve on whole blood, leading to different sample numbers
177 in the 25 and 35°C data sets.

178

179 *Hemoglobin purification and hemoglobin heterogeneity*

180 RBC were shipped on dry ice from Can Tho University to Aarhus University for *in vitro* studies.
181 Water was added and lysed RBC were centrifuged at 8,100 g for 10 min to separate Hb from
182 cellular debris. To strip Hb from allosteric effectors, the hemolysate was dialyzed in a dialysis bag
183 with a 15 kDa cutoff membrane (Spectrum Laboratories, Inc., Roncho Dominguez, Canada) against
184 a 200 times larger volume of 10 mmol l⁻¹ Hepes buffer (pH=7.4) at 4°C. The dialysis buffer was
185 changed 3 times over 24 h. Subsequently, Hb was concentrated by ultrafiltration in Amicon 4 ml-
186 ultrafiltration tubes fitted with a 10 kDa cutoff membrane (Millipore, Tullagren, Ireland) at 4,000 g
187 and stored at -80°C in aliquots at a heme concentration of 7.7 mmol l⁻¹. To evaluate Hb
188 heterogeneity, individual blood samples were prepared as previously described (11) and analyzed
189 by isoelectric focusing (IEF) on thin polyacrylamide gels using a PhastSystem apparatus (GE
190 Healthcare, Uppsala, Sweden) at 15°C. To observe whether temperature acclimation induced
191 expression of alternative Hb isoforms, IEF was conducted on blood from fishes reared at 27 and
192 33°C on long-range polyacrylamide gels (pH-range 3-9). To evaluate the relative expression of the
193 individual isoforms, IEF was conducted on short range polyacrylamide gels (pH-range 5-8) on
194 blood from 27°C acclimated fishes, and the relative expression of the individual Hb bands
195 quantified by densitometric analysis using Image J.

196

197 *Hemoglobin O₂ equilibria*

198 Equilibrium between Hb and O₂ was monitored using a modified diffusion chamber. Two serially-
199 coupled Wösthoff gas mixing pumps (Bochum, Germany) delivered humidified gas mixtures at
200 varying PO₂ by mixing atmospheric air with pure N₂ (>99.998%). Absorbance was monitored at
201 426 nm while gas mixtures equilibrated an ultrathin 4 µl Hb sample with heme concentration 0.6
202 mmol l⁻¹ (57, 59). Absorbance was also measured during equilibration with pure O₂ and N₂ to
203 obtain the full saturation range. Different fractional saturations (*S*) were obtained by stepwise
204 increases in the gas mixture PO₂. pH was adjusted with 0.1 mol l⁻¹ Hepes buffer to obtain Hb-O₂
205 binding curves at 6 different pH values between 6.5-8.5. pH was measured at the experimental
206 temperature with a Mettler Toledo pH/ion meter S220 (Schwerzenbach, Switzerland). To evaluate
207 the influence of ATP and Cl⁻ on Hb oxygenation, and their effects on pH- and temperature
208 sensitivity, O₂ equilibria were measured with and without 100 mmol l⁻¹ KCl and 0.3 mmol l⁻¹ ATP
209 (ATP/Hb₄ = 2 corresponding to the approximate intraerythroid ratio; Table 1) at 15, 20, 25, 30 and
210 35°C (±0.2°C) and at 6 pH-values.

211

212 *Data analysis*

213 Concentration of Hb bound O₂ in blood ([HbO₂]) was calculated by subtracting the physically
214 dissolved O₂ from [O₂].

$$[\text{HbO}_2] = [\text{O}_2] - \alpha_{\text{O}_2} P\text{O}_2$$

215 Where α_{O_2} is O₂ solubility (12), and $P\text{O}_2$ is the $P\text{O}_2$ delivered by the Wösthoff pumps.

216 Fractional O₂ saturation (*S*) for blood was found as [HbO₂] relative to [HbO₂] during
217 equilibration with 30% O₂:

$$S = \frac{[\text{HbO}_2]}{[\text{HbO}_2]_{30\% \text{ O}_2}}$$

218 O₂-affinity (P_{50} : partial pressure of oxygen at half saturation) and cooperativity of O₂
219 binding (n_{50} : the Hill cooperativity coefficient at half saturation) for blood and stripped Hb were
220 determined as the zero intercept and slope of the Hill plot ($\log(S/(1-S))$ vs. $\log P\text{O}_2$), respectively.
221 The Bohr factor, φ , was determined from the slope of Bohr plots ($\log P_{50}$ vs. pH) for both blood and
222 stripped Hb.

$$\varphi = \frac{\partial \log P_{50}}{\partial \text{pH}}$$

223 ΔH_{app} was calculated from the van't Hoff equation for both blood and stripped Hb.

224

$$\Delta H_{\text{app}} = 2.303 R \left(\frac{\partial \log P_{50}}{\partial T^{-1}} \right)$$

225

226 Where the used $\log P_{50}$ values were interpolated from the Bohr plots (Fig.1) at 0.1 pH-value
 227 intervals. ΔH_{app} -values are reported in kcal mol⁻¹ (1 kcal mol⁻¹ = 4.184 kJ mol⁻¹). For Hb, ΔH_{app} was
 228 calculated based on $\log P_{50}$ values at 15, 20, 25, 30 and 35°C, whereas blood ΔH_{app} was calculated
 229 using $\log P_{50}$ values at 25 and 35°C.

230

Mean corpuscular tetrameric Hb concentration (MCHC) was found from the Hct:

$$\text{MCHC} = \frac{[\text{Hb}]}{\text{Hct}}$$

231

P_{50} and ΔH_{app} values for Hb solutions and blood are plotted on intracellular pH (pH_i)
 232 and pH_e axes respectively. The pH_i axis is shifted 0.3 pH units in Fig. 4, to account for the lower
 233 pH_i compared to pH_e, as measured previously in *P. hypophthalmus* (Phuong unpublished). ΔH_{app} is
 234 the sum ΔH^{O_2} , ΔH^{cc} , $\Delta H^{\text{H}_2\text{O}}$ and $\Delta H^{\text{effector}}$, where $\Delta H^{\text{H}_2\text{O}}$ is -3.0 kcal mol⁻¹ (1). All values are
 235 expressed as means ± standard error of mean unless otherwise indicated.

236

237

238

239

RESULTS

240

Arterial blood gases

241

P. hypophthalmus had a high Hct (30±1.4%) and a correspondingly high O₂ carrying capacity of
 242 5.8±1.3 mmol l⁻¹ (Table 1). PCO₂ and PO₂ values were 4.7±0.7 mmHg and 31.8±8.7 mmHg,
 243 respectively, and pH_e was 7.62±0.02 (Table 1) at 27°C.

244

245

Blood tonometry

246

P. hypophthalmus blood bound O₂ cooperatively (n_{50} ~1-3) and with a high O₂ affinity at 25°C
 247 (P_{50} =4.61 mmHg at pH_e 7.6), but with a lower O₂ affinity at 35°C (P_{50} =21.7 mmHg at pH_e 7.6)
 248 (Fig. 1, lower right panel). Blood $\log P_{50}$ values superimposed those of Hb with ATP at 25°C (taking
 249 into account that pH_i is 0.3 pH units lower than pH_e), but were slightly higher at 35°C. The Bohr
 250 factors for blood were -0.70 and -0.39 at 25 and 35°C, respectively (Fig. 2). Decreases in pH_e down
 251 to 6.7 failed to cause a change in blood saturation, showing the absence of a Root effect in
 252 wholeblood (Fig. 3). Blood showed a high temperature sensitivity with $\partial P_{50} / \partial T^{-1}$ =1.71 mmHg °C⁻¹
 253 (ΔH_{app} =-28.3 kcal mol⁻¹ at pH_e 7.6), which was greater than for stripped Hb (Fig. 4). RBC [NTP]

254 remained constant during the 3 hour equilibration period in the tonometer and was unaffected by
255 heating or cooling the blood sample to temperatures between 25 and 35°C (data not shown).

256

257 *Hemoglobin heterogeneity*

258 Long-range IEF showed one Hb band in both the 27°C and 33°C acclimated groups (not shown).

259 Short-range IEF of the blood of the 27°C acclimated fishes revealed 6 distinct electrophoretic

260 bands, revealing anodic Hb isoforms with closely similar isoelectric points (pI) (7.44-7.67) (Fig. 5).

261

262 *Hemoglobin oxygen equilibria*

263 Evaluation of O₂ binding in stripped *P. hypophthalmus* Hb revealed high cooperativity ($n_{50} \sim 2.5$)

264 and high affinity ($P_{50} = 5.9$ mmHg at pHi 7.3 and 25°C, Fig. 1). Cooperativity remained high over

265 the whole experimental pHi-range (from above pHi 8 to below pHi 6.5 (Fig. 1), supporting the

266 absence of a Root effect for the Hb. Addition of 100 mmol l⁻¹ KCl did not affect Hb oxygenation,

267 whereas addition of ATP decreased Hb-O₂ affinity slightly at lowered pHi (Fig. 1, 4).

268 The Bohr factors of Hb and blood, decreased at higher temperatures (Fig. 2). Addition

269 of ATP increased the Bohr factor markedly, whereas addition of Cl⁻ had little effect (Fig. 2). The

270 Bohr factor for blood at 35°C was similar to Hb with ATP, but was slightly higher at 25°C. To

271 evaluate the temperature and pH sensitivities of ATP and Cl⁻ binding, O₂ equilibrium curves were

272 measured at 5 temperatures to determine ΔH_{app} . ΔH_{app} for stripped Hb was -16.18 kcal mol⁻¹

273 corresponding to -13.18 kcal mol⁻¹ for ΔH^{O_2} and ΔH^{cc} (by subtracting -3.0 kcal mol⁻¹ for ΔH^{H_2O}

274 (1)), which is similar to that of human Hb (2). In agreement with Fig. 1, Cl⁻ did not bind to

275 oxygenation-linked binding sites on Hb, producing a near zero ΔH^{Cl^-} (Fig. 4). In the presence of

276 ATP, Hb showed a consistently lower temperature sensitivity of oxygenation, revealing

277 endothermic release of bound ATP (and associated extra Bohr protons) amounting to 3.1 kcal mol⁻¹.

278

279

DISCUSSION

280 *Blood oxygen binding*

281 *P. hypophthalmus* blood bound O₂ with an unusually high affinity at 25°C, when compared to most

282 other air-breathing fishes studied to date (Table 2). Most of these species have an inactive lifestyle,

283 whereas *P. hypophthalmus* is a fast swimmer with high maximal rates of O₂ uptake (34). Hence, the

284 high O₂ affinity appears unfavorable in terms of O₂ unloading to the tissues. The high O₂ affinity

285 seems beneficial in relation to branchial O₂ uptake during mild environmental hypoxia (55) and in

286 terms of limiting branchial O₂ loss during severe hypoxia/anoxia, where *P. hypophthalmus* becomes
287 more reliant on aerial O₂ uptake (33). While a high O₂ affinity aids in O₂ uptake, the constrained O₂
288 unloading in tissue capillaries may require a high capillary density to reduce the diffusive distance
289 from capillaries to cells (10, 55). A high O₂ flux to the tissues concurrent with high O₂ affinity can
290 be achieved through a large Bohr/Root effect (47, 48), high O₂ carrying capacity of blood, high
291 perfusion and/or a high O₂ diffusive capacity of the tissues (21). The magnitude of the Bohr effect
292 of *P. hypophthalmus* blood is similar to other air-breathing fishes (Table 2) and thus contributes
293 small increases in blood PO₂ as RBCs passes tissue capillaries, and a relatively high O₂ carrying
294 capacity of the blood may serve as a trait to increase systemic O₂ delivery, as observed in the
295 swamp eel, *Monopterus albus* (10). It would be interesting to devote future studies to capillary
296 density measurements in this species.

297 n_{50} values were generally higher in Hb solutions compared to blood. The Hb solutions
298 were highly buffered, whereas oxygenation linked H⁺ dissociation decreases pH_i during
299 oxygenation in whole blood, resulting in lower apparent n_{50} values in blood compared to Hb (23), as
300 has been observed previously (10, 41).

301 The absence of a Root effect in *P. hypophthalmus* was demonstrated in whole blood
302 (Fig. 3) and was supported in Hb solutions by the complete lack of any loss of cooperativity at low
303 pH (Fig. 1). The absence of Root effect in *P. hypophthalmus*, and its apparent lack of β -
304 adrenergically stimulated Na⁺/H⁺ exchange (β_{NHE}) (Phuong, unpublished), is in line with the
305 reduction in the Root effect in the ancestor of Siluriformes, as well as the reduction in β_{NHE} activity
306 in the ancestor of Siluriformes/Gymnotiformes fishes after the divergence from Characiformes
307 fishes (4). *P. hypophthalmus* inhabits tropical freshwater environments that may become severely
308 hypercapnic, and a lack of Root effect may serve to maintain a high O₂ carrying capacity during
309 hypercapnia. In contrast, a Root effect is a requirement for O₂ secretion from a choroid rete to
310 generate the high PO₂ necessary to drive oxygen across the long diffusion distance of the avascular
311 retina of most fishes (56). However, in contrast to other fishes with secondary reductions in the
312 magnitude of the Root effect (4), *P. hypophthalmus* responds strongly to visual stimuli and is
313 clearly capable of matching O₂ supply with O₂ demands of the retina. Thus, future studies must
314 examine the anatomical arrangements and function of the ocular vasculature, and identify eventual
315 vascularization of the retina, as observed *e.g.* in eel (56).

316 Blood O₂-binding in *P. hypophthalmus* was strongly temperature dependent, such that
317 O₂ affinity falls markedly more with increased temperature than in other air-breathing fishes (Table

318 2). Unexpectedly, the temperature effect for blood is higher than for Hb (Fig. 4), which cannot be
319 explained by this dataset, as this would require oxygenation-linked association of allosteric
320 effectors in the order of 16 kcal mol^{-1} . The higher temperature sensitivity seems to be a sum of two
321 factors. First, the apparently lower O_2 affinity for blood compared to Hb at 35°C translates into a
322 higher ΔH_{app} for blood compared to Hb. Second, blood P_{50} values show higher variation compared
323 to Hb P_{50} values, which translates into a broad ΔH_{app} confidence interval for blood, which overlaps
324 with the Hb ΔH_{app} plot (not shown). Therefore, the absolute ΔH_{app} -value for blood should be taken
325 with caution. However, both approaches in our dataset confirm the overall conclusion that O_2
326 binding in *P. hypophthalmus* is unusually high and results from reduced interactions between
327 allosteric effectors and Hb.

328 The adaptive significance of a high temperature sensitivity of blood O_2 binding is
329 unknown. *P. hypophthalmus* is distributed throughout the Mekong River in water with little
330 variation in temperatures (38), as is also the case in aquatic habitats of the African lungfish, which
331 also has blood with a high temperature sensitivity (36). The Australian lungfish, in contrast,
332 experiences large temperature fluctuations and has less temperature sensitive blood (37). Following
333 this analogy, a high temperature sensitivity might be a tolerable trait in fishes living in stenothermal
334 environments. While the adaptive significance (if any) of a high temperature sensitivity remains
335 unclear, it might be simply a thermodynamic consequence of the reduced allosteric effector binding.

336

337 *Molecular interpretation of temperature effect and high affinity*

338 We demonstrated that the Hb has low sensitivity to ATP above pH_i 7.4 and to Cl^- ions over the
339 whole physiological pH -range (Fig. 1, 4). Both anions would normally stabilize Hb in its low O_2 -
340 affinity tense state conformation and thus lower the Hb- O_2 affinity (44). The weak oxygenation-
341 linked anion binding thus only decreases O_2 affinity slightly below the intrinsic Hb- O_2 affinity, and
342 thereby provides a potent mechanism for increasing blood O_2 affinity above normal. A similar
343 adaptation has been observed in two other hypoxia adapted aquatic vertebrates. Hb of the Andean
344 frog *Telmatobius peruvianus*, inhabiting mountain lakes above 3,800 m is insensitive to Cl^- (60, 66)
345 as is Hb of the hypoxia tolerant swamp eel (10) resulting in a high blood O_2 affinity in both cases.
346 Evolution of Hb with reduced Cl^- insensitivity thus seems to be a common mechanism for
347 efficiently increasing blood O_2 affinity in response to hypoxia.

348 ATP binds to Hb in the physiological pH_i -range, but only decreases Hb- O_2 affinity
349 below approximately pH_i 7.4 (Fig. 1, 4). This contrasts to the normal trend, where ATP decreases

350 O₂ affinity over a larger pH_i-range (11, 15, 66). During environmental hypoxia intra-erythroid ATP
351 concentration decreases in many ectothermic vertebrates, and this is associated with decreased
352 inhibitory interactions of ATP on Hb, which increases the blood O₂ affinity during environmental
353 hypoxia (51, 54, 65, 68). The reduced ability for ATP to decrease O₂ affinity in *P. hypophthalmus*
354 Hb consequently only allows for a small degree of hypoxia-induced increase in blood O₂ affinity.
355 GTP is present in RBC of many fishes, including some siluriform fishes and can exert an effect on
356 Hb O₂ affinity in some species (3, 22, 53, 65). Given the weak binding of ATP to Hb as well as
357 comparable O₂ affinities for blood and Hb at 25°C, we can assume that GTP does not exert
358 significant effects on Hb-O₂ affinity.

359 Hb O₂ binding is exothermic, and normally in vertebrate Hbs, oxygenation is linked to
360 an endothermic release of Cl⁻, organic phosphates and protons, off-setting the exothermy of
361 oxygenation (63) and thereby reducing the temperature sensitivity of O₂ binding. Oxygenation-
362 linked ATP-binding is much weaker in *P. hypophthalmus* compared to other species as evident in
363 the low ΔH^{ATP} (3.08 kcal mol⁻¹ versus 9.9 - 21 kcal mol⁻¹ reported in other species) and the low
364 reduction in O₂ affinity upon ATP addition (25, 62). Thus, the lack of oxygen binding modulation
365 by both ATP and Cl⁻ combine in *P. hypophthalmus* blood leaving the exothermy of O₂ binding in
366 Hb intact and imparting a high temperature effect on blood O₂ affinity.

367 Temperature acclimation to 27°C and 33°C revealed no changes in Hb isoforms, as
368 only one Hb band was found on the long-range IEF gels. Short-range IEF of blood at 27°C revealed
369 expression of six anodic Hb isoform components in *P. hypophthalmus* with very similar pI. The
370 functional significance of co-expression of multiple Hb isoforms with distinct functional properties
371 has been hypothesized to provide functional division of labor in gas transport between the Hb
372 isoform components, so O₂ uptake and delivery can function over broader ranges of environmental
373 factors (e.g. PO₂, pH, temperature) (19, 58, 63, 65, 67). However, no studies have documented its
374 direct physiological benefits.

375

376 *Perspectives and significance*

377 This study documents how a lack of Cl⁻ binding and weak ATP binding to Hb is associated with a
378 high blood O₂ affinity related to inhabiting freshwater environments that are frequently very
379 hypoxic. The weak anion sensitivity of Hb is found in other hypoxia-adapted aquatic vertebrates
380 and allows for high blood O₂ affinity. Weak anion sensitivity of Hb limits the modulation of O₂
381 affinity through changes in RBC organic phosphate concentration, and is associated with chronic

382 high O₂ affinity. An inevitable consequence is a high temperature sensitivity of blood due to this
383 lack of oxygenation-linked endothermic release of anionic effectors, and this study documents the
384 highest temperature sensitivity of O₂ binding measured in blood of an air-breathing fish. This
385 suggests that at low temperatures, O₂ unloading may be compromised by a high O₂ affinity, limiting
386 O₂ transport, whereas higher temperatures may constrain O₂ uptake. It will therefore be of interest
387 to conduct future studies on the aerobic performance across temperatures in this species to clarify if
388 this common hypoxia-adaptation is associated with a narrow optimal temperature range as well as a
389 high optimal temperature for aerobic scope.

390

391

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DISCLOSURES

401 The authors report no conflict of interest.

402

403

AUTHOR CONTRIBUTIONS

404 C.D performed experiments, C.D. analyzed data and prepared figures, C.D., T.W. and M.B.
405 designed the study; C.D., F.B.J., T.W. and M.B. interpreted results and drafted, edited, and revised
406 manuscript; all authors approved final version of manuscript.

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586

587

FIGURE CAPTIONS

588 Fig. 1. pH-dependencies of n_{50} and P_{50} of *P. hypophthalmus* Hb and blood at the 5 experimental
589 temperatures (for blood only 25°C and 35°C) in the absence of allosteric effectors (black symbols)
590 and in the presence of 100 mmol l⁻¹ KCl (blue symbols) or 3 mmol l⁻¹ ATP (ATP/Hb = 2) (green
591 symbols) and in blood (red symbols).

592

593 Fig. 2. pH-dependency of O₂ binding (Bohr factor (ϕ)) at the 5 experimental temperatures in the
594 absence (black bars) and presence of 100 mmol l⁻¹ KCl (blue bars) and 3 mmol l⁻¹ ATP (green bars)

595 as well as in blood (red bars). Bars with error bars indicate slope of the Bohr plots + standard error
 596 of estimated slope.

597

598 Fig. 3. Blood O₂ saturation during progressive increases in P_{CO₂} (3-230 mmHg) during
 599 equilibration with air. The lack of a reduction in blood O₂ saturation at lowered pH documents the
 600 absence of a Root effect.

601

602 Fig. 4. Temperature sensitivity of O₂ binding presented as the apparent heat of oxygenation (ΔH_{app})
 603 in the absence (black line) and presence of 100 mmol l⁻¹ KCl (blue line) and 0.3 mmol l⁻¹ ATP
 604 (green line) as well as in blood (red line). Solid and dotted lines indicate mean and standard error of
 605 estimated ΔH_{app} -values, respectively. Data for Hb and blood are plotted on intracellular- and
 606 extracellular pH axes, respectively.

607

608 Fig. 5. Relative abundance and isoelectric points of the six Hb isoforms in *Pangasianodon*
 609 *hypophthalmus* red blood cells. Data are mean \pm s.e.m. (n=10).

610

611

612

TABLES

613 Table 1. Arterial values for hematocrit (Hct), mean corpuscular tetrameric hemoglobin
 614 concentration (MCHC), red blood cell nucleotide triphosphate concentration
 615 ($[NTP]=[ATP]+[GTP]$), partial pressure of CO₂ and O₂ (P_{CO₂} and P_{O₂}) and extracellular pH
 616 (pH_e) in *Pangasianodon hypophthalmus* at 27°C.

Hct [%]	30±1.4 (12)
O ₂ carrying capacity [mmol l ⁻¹]	5.8±1.3 (11)
MCHC [mmol l ⁻¹]	5.46±1.4 (11)
NTP [mmol l _{RBC} ⁻¹]	7.04±0.25 (11)
[NTP]/MCHC	1.44±0.23 (9)
P _{CO₂} [mmHg]	4.65±0.7 (12)
P _{O₂} [mmHg]	31.8±8.7 (4)
pH _e	7.62±0.02 (12)

617 Values are mean \pm standard error of mean. Values in parenthesis indicate number of replicates.

618

619 Table 2. Comparison of oxygen affinities (P_{50}), Bohr effects (ϕ) and apparent heat of oxygenation (ΔH_{app}) in blood of air-breathing fishes.

620

Species	Air-breathing structure	Blood P_{50} [mmHg]	ϕ	ΔH_{app} [kcal mol ⁻¹]	Conditions	Reference
Class: Actinopterygii						
Order: Ammiformes						
Family: Amiidae						
<i>Amia calva</i>	Air-bladder	24.0	ND	-16.5	27°C, pH 7.6	(26)
Order: Lepisosteiformes						
Family: Lepisosteidae						
<i>Lepisosteus oculatus</i>	Lung	24.1	-0.5	ND	20°C, PCO ₂ 7 mmHg	(49)
Order: Osteoglossiformes						
Family: Arapaimidae						
<i>Arapaima gigas</i>	Swim-bladder	21.0	-0.30	ND	28°C, pH 7.4	(30)
Order: Gymnotiformes						
Family: Gymnotidae						
<i>Electrophorus electricus</i>	Buccopharyngeal cavity	10.7	-0.78	ND	28°C, pH 7.6	(28)
Order: Characiformes						
Family: Erythrinidae						
<i>Hoplerethrinus unitaeniatus</i>	Swim-bladder	11.4	-0.75	-21.4	30°C	(46)
Order: Siluriformes						
Family: Loricariidae						
<i>Pterygoplichthys multiradiatus</i>	Stomach	4.41	-0.13	ND	25°C, pH 7.6	(54)
<i>Ancistrus chagresi</i>	Stomach	19.8	ND	ND	25°C, pH 7.4	(16)
Family: Callichthyidae						
<i>Hoplosternum littorale</i>	Intestine	9.5	-0.33	ND	30°C	(46)
Family: Pangasiidae						
<i>Pangasianodon hypophthalmus</i>	Swim-bladder	4.6	-0.70	-28.3	25°C, pH 7.6	This study
Family: Ictaluridae						
<i>Ictalurus nebulosus</i>	Swim-bladder	10.2	-0.45	-12.8	24°C, pH 7.6	(18)
Order: Esociformes						
Family: Umbridae						
<i>Dallia pectoralis</i> *	Oesophagus	10.5	ND	-11.3	15°C, pH 7.4	(32)

Order: Synbranchiformes						
Family: Symbranchidae						
<i>Monopterus albus</i>	Buccopharyngeal cavity	4.1	-0.79	ND	27°C, pH 7.5	(10)
<i>Monopterus cuchia</i>	Buccopharyngeal cavity	7.9	-0.57	-13.1	30°C, pH 7.6	(39)
<i>Synbranchus marmoratus</i>	Buccopharyngeal cavity	7.0	-0.69	ND	30°C, pH 7.8	(20)
Order: Perciformes						
Family: Channidae						
<i>Channa maculate</i>	Suprabranchial organ	7.6	-0.70	ND	25°C, pH 7.6	(69)
Class: Sarcopterygii						
Order: Lepidosireniformes						
Family: Protopteridae						
<i>Protopterus aethiopicus</i>	Lung	10.0	-0.47	ND	25°C, PCO ₂ 6 mmHg	(36)
Family: Lepidosirenidae						
<i>Lepidosiren paradora</i>	Lung	7.1	-0.24	ND	23°C, PCO ₂ 6 mmHg	(27)
Order: Caratodontiformes						
Family: Caratodontidae						
<i>Neoceratodus fosteri</i>	Lung	11	-0.62	ND	18°C, PCO ₂ 3.5 mmHg	(37)









