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

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**Glossary**

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<th>Term</th>
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<td>pH2</td>
<td>Extracellular pH</td>
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<tr>
<td>pHI</td>
<td>Intracellular pH</td>
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<tr>
<td>pI</td>
<td>Isoelectric point</td>
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<tr>
<td>R</td>
<td>Ideal gas constant</td>
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<td>S</td>
<td>Red blood cell</td>
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<tr>
<td>S02</td>
<td>Fractional oxygen saturation</td>
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<tr>
<td>Sapp</td>
<td>Solubility coefficient of oxygen</td>
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<td>βSHE</td>
<td>β-adrenergically stimulated Na+/H+ exchange</td>
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<tr>
<td>ΔHapp</td>
<td>Apparent enthalpy of oxygenation</td>
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<tr>
<td>ΔHCC</td>
<td>Enthalpy of oxygenation-linked conformational changes</td>
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<td>ΔH2O</td>
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<td>ΔH2O2</td>
<td>Intrinsic enthalpy of heme oxygen binding</td>
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<td>φ</td>
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**THE SOUTHEAST 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 Southeast 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 and high temperature sensitivity of blood O2 binding. This study demonstrates how a potent mechanism for increasing O2 affinity is linked to increased temperature sensitivity of O2 transport and provides a basic framework for a better understanding of how hypoxia-adapted species will react to increasing temperatures.

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incorporated into functionally distinct Hb tetramers (52). 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 PO2 (Root effect) (24). This property enables O2 offloading to a very high P02 and, hence, swim bladder filling at depth and oxygenation of ocular and other tissues (4, 47, 48). Furthermore, fish Hbs normally bind RBC more effectively than their human counterparts, and their O2 loadings are for example, be safeguarded to the extremities of Arctic mammals (7, 8, 15), and where O2 delivery can be regulated to be maintained by flushing the gills with water containing 0.05 g benzocaine/l. When immobile, the fishes were transferred to a surgical table, where anesthesia was maintained by flushing the gills with water containing 0.05 g benzocaine/l. A PE-50 catheter was inserted into the dorsal aorta through the mouth and extended through a hole in the rostrum (50). The catheter was secured to the dorsal roof of the mouth and at the dorsal side of the fish by sutures, and the fish was allowed to recover for 24–72 h in normoxic water at 27°C, while the catheters were flushed daily with heparinized saline. For blood measurements, a sample of up to 10 ml was taken to determine arterial blood gas tensions and to construct blood O2 equilibrium curves in vitro. All experiments were performed in accordance with national guidelines for the protection of animal welfare in Vietnam.

Arterial blood parameters. Arterial PO2 and extracellular pH (pHe) were measured in each fish using a GEM Premier 3500 automated blood gas analyzer (Instrumentation Laboratory, Bedford, MA) (40). At P02 of 115 and 230 mmHg (16 and 32%), pHe was extrapolated from a logP02 vs. pH plot due to the inability of the blood analyzer to measure pHe below 6.8. Arterial PO2 was measured in four fishes using a Radiometer oxygen electrode thermostatted at 27°C and connected to a PHM 71 (Radiometer, Copenhagen, Denmark). Hct was found as the fractional RBC volume after centrifugation at 12,000 rpm for 3 min, and blood tetrameric Hb concentration ([Hb]) was determined spectrophotometrically after conversion to cyanometHb using Drabkin’s reagent. Erythrocyte hematocrit (Hct) was found as the fractional RBC volume after centrifugation at 12,000 rpm for 3 min, and blood tetrameric Hb concentration ([Hb]) was determined spectrophotometrically after conversion to cyanometHb using Drabkin’s reagent. Erythrocyte hematocrit was measured spectrophotometrically (Cecil CE2041, Cambridge, UK) via enzyme-coupled reactions (Sigma Bulletin, no. 366-UV), using neutralized supernatants from blood deproteinized in 12% trichloroacetic acid. Whole blood [NTP] was converted to erythrocyte [NTP] via the corresponding Hct.

Blood tonometry for determination of whole blood oxygen binding properties. Freshly drawn blood was divided into two Eschweiler (Kiel, Germany) tonometers and equilibrated with humidified gas mixtures delivered from serially linked Wösthoff (Bochum, Germany) gas mixing pumps. For blood O2 equilibrium curves, each tonometer was equilibrated with 3.8 or 22.8 mmHg CO2 (0.5 or 3%) at either 25 or 35°C. Blood was equilibrated with 30% O2 to determine the O2 carrying capacity, whereupon P02 was lowered to achieve O2 saturations between 10 and 90%. At each equilibration step, the blood was allowed to equilibrate with the gas for ~30 min, and blood [O2] was measured in duplicate with the Tucker method (52). To quantify the Root effect, blood O2 saturation was measured at 25°C, while equilibrated with air during progressive increases in P02 from 3.8 to 243 mmHg (5% to 32%). In some fish with a smaller blood volume, it was only possible to perform one O2 equilibrium curve on whole blood, leading to different sample numbers in the 25°C and 35°C data sets.

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Hemoglobin purification and hemoglobin heterogeneity. RBC were shipped on dry ice from Can Tho University to Aarhus University for in vitro studies. Water was added and lysed RBC were centrifuged at 8,100 g for 10 min to separate Hb from cellular debris. To strip Hb from allosteric effectors, the hemolysate was dialyzed in a dialysis bag with a 15-kDa cutoff membrane (Spectrum Laboratories, Roncho
Oxygen binding of Pangasius blood and hemoglobin

DOmiguez, Canada) against a 200 times larger volume of 10 mmol/l HEPES buffer (pH = 7.4) at 4°C. The dialysis buffer was changed 3 times over 24 h. Subsequently, Hb was concentrated by ultrafiltration in Amicon 4-mL-ultrafiltration tubes fitted with a 10-kDa cutoff membrane (Millipore, Tullagren, Ireland) at 4,000 g and stored at −80°C in aliquots at a heme concentration of 7.7 mmol/l. To evaluate Hb heterogeneity, individual blood samples were prepared as previously described (11) and analyzed by isoelectric focusing (IEF) on thin polyacrylamide gels using a PhastSystem apparatus (GE Healthcare, Uppsala, Sweden) at 15°C. To observe whether temperature acclimation induced expression of alternative Hb isoforms, IEF was conducted on blood from fishes reared at 27°C and 33°C on long-range polyacrylamide gels (pH range: 3–9). To evaluate the relative expression of the individual isoforms, IEF was conducted on short-range polyacrylamide gels (pH range: 5–8) on blood from 27°C-acclimated fishes, and the relative expression of the individual Hb bands was quantified by densitometric analysis using ImageJ software.

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

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

\[
[HbO2] = [O2] - \alpha_{O2}PO2
\]

where \(\alpha_{O2}\) is O2 solubility (12), and PO2 is the PO2 delivered by the Wösthoff pumps. Fractional O2 saturation (S) for blood was found as [HbO2] relative to [HbO2] during equilibration with 30% O2:

\[
S = \frac{[HbO2]}{[HbO2]_{30\%_O2}}
\]

where the used logP50 values were interpolated from the Bohr plots (Fig. 1) at 0.1 pH-value intervals. \(\Delta H_{app}\) values are reported in kcal/mol (1 kcal/mol = 4.184 KJ/mol). For Hb, \(\Delta H_{app}\) was calculated on the basis of logP50 values at 15°C, 20°C, 25°C, 30°C, and 35°C, whereas blood \(\Delta H_{app}\) was calculated using logP50 values at 25°C and 35°C.

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

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

Arterial blood gases. P. hypophthalmus had a high Hct (30 ± 1.4%) and a correspondingly high O2 carrying capacity of 5.8 ± 1.3 mmol/l (Table 1). Pco2 and Pco2 values were 4.7 ± 0.7 mmHg and 31.8 ± 8.7 mmHg, respectively, and pH was 7.62 ± 0.02 (Table 1) at 27°C.

Blood tonometry. P. hypophthalmus blood bound O2 cooperatively (\(K_{50}\) ≈1–3) and with a high O2 affinity at 25°C (\(P_{50} = 4.61 \text{ mmHg at } pH_7.6\)), but with a lower O2 affinity at 35°C (\(P_{50} = 21.7 \text{ mmHg at } pH_7.6\)) (Fig. 1, lower right panel). Blood logP50 values superimposed those of Hb with ATP at 25°C (taking into account that pH is 0.3 pH units lower than pH7.6) but were slightly higher at 35°C. The Bohr factors for blood were −0.70 and −0.39 at 25°C and 35°C, respectively (Fig. 2). Decreases in pH4 down to 6.7 failed to cause a change in blood saturation, showing the absence of a Root effect in whole blood (Fig. 3). Blood showed a high temperature sensitivity with \(\frac{\partial P_{50}}{\partial T^{-1}} = 1.71 \text{ mmHg°C}^{-1}\) (\(\Delta H_{app} = -28.3 \text{ kcal/mol at } pH_7.6\)), which was greater than for stripped Hb (Fig. 4). RBC [NTP] remained constant during the 3-h equilibration period in the tonometer and was unaffected by heating or cooling the blood sample to temperatures between 25°C and 35°C (data not shown).

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

**Table 1. Arterial values for hematocrit, mean corpuscular tetrameric hemoglobin concentration, red blood cell nucleotide triphosphate concentration, partial pressure of CO2 and O2, and extracellular pH in Pangasianodon hypophthalmus at 27°C**

<table>
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<tr>
<th>Arterial Parameters</th>
<th>Value</th>
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<tr>
<td>Hct, %</td>
<td>30 ± 1.4 (12)</td>
</tr>
<tr>
<td>O2 carrying capacity, mmol/l</td>
<td>5.8 ± 1.3 (11)</td>
</tr>
<tr>
<td>MCHC, mmol/l</td>
<td>5.46 ± 1.4 (11)</td>
</tr>
<tr>
<td>NTP, mmol/l intrac</td>
<td>7.04 ± 0.25 (11)</td>
</tr>
<tr>
<td>[NTP]/MCHC</td>
<td>1.44 ± 0.23 (9)</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>4.65 ± 0.7 (12)</td>
</tr>
<tr>
<td>Pco2, mmHg</td>
<td>31.8 ± 8.7 (4)</td>
</tr>
<tr>
<td>pH4</td>
<td>7.62 ± 0.02 (12)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Values in parenthesis indicate number of replicates. Hct, hematocrit; MCHC, mean corpuscular tetrameric hemoglobin concentration; NTP, nucleotide triphosphate concentration ([NTP] = [ATP] + [GTP]); pH4, extracellular pH.

**RESULTS**

**Arterial blood gases.** P. hypophthalmus had a high Hct (30 ± 1.4%) and a correspondingly high O2 carrying capacity of 5.8 ± 1.3 mmol/l (Table 1). Pco2 and Pco2 values were 4.7 ± 0.7 mmHg and 31.8 ± 8.7 mmHg, respectively, and pH was 7.62 ± 0.02 (Table 1) at 27°C.

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shown). Short-range IEF of the blood of the 27°C-acclimated fishes revealed six distinct electrophoretic bands, revealing anodic Hb isoforms with closely similar isoelectric points (pI) (7.44 – 7.67) (Fig. 5).

Hemoglobin oxygen equilibria. Evaluation of O₂ binding in stripped P. hypophthalmus Hb revealed high cooperativity (n ≈ 2.5) and high affinity (P₅₀ = 5.9 mmHg at pH 7.3 and 25°C; Fig. 1). Cooperativity remained high over the whole experimental pH range (from above pH 8 to below pH 6.5) (Fig. 1), supporting the absence of a Root effect for the Hb. The addition of 100 mmol/l KCl did not affect Hb oxygenation, whereas the addition of ATP decreased Hb-O₂ affinity slightly at lowered pH (Figs. 1 and 4).

The Bohr factors of Hb and blood decreased at higher temperatures (Fig. 2). The addition of ATP increased the Bohr factor markedly, whereas the addition of Cl⁻ had little effect (Fig. 2). The Bohr factor for blood at 35°C was similar to Hb with ATP, but was slightly higher at 25°C. To evaluate the temperature and pH sensitivities of ATP and Cl⁻ binding, O₂ equilibrium curves were measured at five temperatures to determine ΔH_app. ΔH_app for stripped Hb was −16.18 kcal/mol corresponding to −13.18 kcal/mol for ΔH⁰² and ΔHᶠᶜ [by subtracting −3.0 kcal/mol for ΔHᐟ²[H₂O] (1)], which is similar to that of human Hb (2). In agreement with Fig. 1, Cl⁻ did not bind to oxygenation-linked binding sites on Hb, producing a near zero ΔH_Cl⁻ (Fig. 4). In the presence of ATP, Hb showed a consistently lower temperature sensitivity of oxygenation, revealing endothermic release of bound ATP (and associated extra Bohr protons) amounting to 3.1 kcal/mol.

DISCUSSION

Blood oxygen binding. P. hypophthalmus blood bound O₂ with an unusually high affinity at 25°C, compared with most other air-breathing fishes studied to date (Table 2). Most of these species have an inactive lifestyle, whereas P. hypophthalmus is a fast swimmer with high maximal rates of O₂ uptake (34). Hence, the high O₂ affinity appears unfavorable in terms of O₂ unloading to the tissues. The high O₂ affinity seems beneficial in relation to branchial O₂ uptake during mild environmental hypoxia (55) and in terms of limiting branchial O₂ loss during severe hypoxia/anoxia, where P. hypophthalmus becomes more reliant on aerial O₂ uptake (33). While a high O₂ affinity aids in O₂ uptake, the constrained O₂ unloading in tissue capillaries may require a high capillary density to reduce the diffusive distance from capillaries to cells (10, 55). A high O₂ flux to the tissues concurrent with high O₂ affinity can be achieved through a large Bohr/Root effect (47, 48), high O₂ carrying capacity of blood, high perfusion and/or a high O₂ diffusive capacity of the tissues (21). The magnitude of the
Bohr effect of *P. hypophthalmus* blood is similar to other air-breathing fishes (Table 2) and, thus, contributes small increases in blood PO$_2$ as RBCs pass through tissue capillaries, and a relatively high O$_2$ carrying capacity of the blood may serve as a trait to increase systemic O$_2$ delivery, as observed in the swamp eel, *Monopterus albus* (10). It would be interesting to devote future studies to capillary density measurements in this species.

$n_{50}$ values were generally higher in Hb solutions compared with blood. The Hb solutions were highly buffered, whereas oxygenation-linked H$^+$ dissociation decreases pH$_i$ during oxygenation in whole blood, resulting in lower apparent $n_{50}$ values in blood compared with Hb (23), as has been observed previously (10, 41).

The absence of a Root effect in *P. hypophthalmus* was demonstrated in whole blood (Fig. 3) and was supported in Hb solutions by the complete lack of any loss of cooperativity at low pH (Fig. 1). The absence of the Root effect in *P. hypophthalmus* and its apparent lack of β-adrenergically stimulated Na$^+$/H$^+$ exchange (β$_{NHE}$) (Phuong LM, unpublished data) is in line with the reduction in the Root effect in the ancestor of Siluriformes, as well as the reduction in β$_{NHE}$ activity in the ancestor of Siluriformes/Gymnotiformes fishes after the divergence from Characiformes fishes (4). *P. hypophthalmus* inhabits tropical freshwater environments that may become severely hypercapnic, and a lack of Root effect may serve to maintain a high O$_2$ carrying capacity during hypercapnia. In contrast, a Root effect is a requirement for O$_2$ secretion from a choroid rete to generate the high PO$_2$ necessary to drive oxygen across
the long diffusion distance of the avascular retina of most fishes (56). However, in contrast to other fishes with secondary reductions in the magnitude of the Root effect (4), *P. hypophthalmus* responds strongly to visual stimuli and is clearly capable of matching O₂ supply with O₂ demands of the retina. Thus, future studies must examine the anatomical arrangements and function of the ocular vasculature and identify eventual vascularization of the retina, as observed, for example, in eel (56).

Blood O₂-binding in *P. hypophthalmus* was strongly temperature-dependent, such that O₂ affinity falls markedly more with increased temperature than in other air-breathing fishes (Table 2). Unexpectedly, the temperature effect for blood is higher than for Hb (Fig. 4), which cannot be explained by this data set, as this would require oxygenation-linked association of allosteric effectors in the order of 16 kcal/mol. The higher temperature sensitivity seems to be a sum of two factors. First, the apparently lower O₂ affinity for blood compared with Hb at 35°C translates into a higher /H9004Happ for blood compared with Hb. Second, blood P50 values show higher variation compared with Hb P50 values, which translates into a broad /H9004Happ confidence interval for blood, which overlaps with the Hb /H9004Happ plot (not shown). Therefore, the absolute /H9004Happ value for blood should be taken with caution. However, both approaches in our data set confirm the overall conclusion that O₂ binding in *P. hypophthalmus* is unusually high and results from reduced interactions between allosteric effectors and Hb. The adaptive significance of a high-temperature sensitivity of blood O₂ binding is unknown.

### Table 2. Comparison of oxygen affinities (P50), Bohr effects (φ), and apparent heat of oxygenation (ΔHapp) in the blood of air-breathing fishes

<table>
<thead>
<tr>
<th>Species</th>
<th>Air-Breathing Structure</th>
<th>Blood P50, mmHg</th>
<th>φ</th>
<th>ΔHapp, kcal/mol</th>
<th>Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amia calva</em></td>
<td>Air-bladder</td>
<td>24.0</td>
<td>ND</td>
<td>−16.5</td>
<td>27°C, pH 7.6</td>
<td>(26)</td>
</tr>
<tr>
<td><em>Lepisosteus oculatus</em></td>
<td>Lung</td>
<td>24.1</td>
<td>−0.5</td>
<td>ND</td>
<td>20°C, PCO₂ 7 mmHg</td>
<td>(49)</td>
</tr>
<tr>
<td><em>Arapaima gigas</em></td>
<td>Swim-bladder</td>
<td>21.0</td>
<td>−0.30</td>
<td>ND</td>
<td>28°C, pH 7.4</td>
<td>(30)</td>
</tr>
<tr>
<td><em>Electrophorus electricus</em></td>
<td>Buccopharyngeal cavity</td>
<td>10.7</td>
<td>−0.78</td>
<td>ND</td>
<td>28°C, pH 7.6</td>
<td>(28)</td>
</tr>
<tr>
<td><em>Hoplerythrinus unitaeniatus</em></td>
<td>Swim-bladder</td>
<td>11.4</td>
<td>−0.75</td>
<td>−21.4</td>
<td>30°C</td>
<td>(46)</td>
</tr>
<tr>
<td><em>Pangasianodon hypophthalmus</em></td>
<td>Stomach</td>
<td>4.6</td>
<td>−0.70</td>
<td>−28.3</td>
<td>25°C, pH 7.6</td>
<td>(46)</td>
</tr>
<tr>
<td><em>Ictalurus nebulosus</em></td>
<td>Swim-bladder</td>
<td>10.2</td>
<td>−0.45</td>
<td>−12.8</td>
<td>24°C, pH 7.6</td>
<td>(18)</td>
</tr>
<tr>
<td><em>Dallia pectoralis</em></td>
<td>Esophagus</td>
<td>10.5</td>
<td>ND</td>
<td>−11.3</td>
<td>15°C, pH 7.4</td>
<td>(32)</td>
</tr>
<tr>
<td><em>Monopterus albus</em></td>
<td>Buccopharyngeal cavity</td>
<td>4.1</td>
<td>−0.79</td>
<td>ND</td>
<td>27°C, pH 7.5</td>
<td>(10)</td>
</tr>
<tr>
<td><em>Monopterus cuchia</em></td>
<td>Buccopharyngeal cavity</td>
<td>7.9</td>
<td>−0.57</td>
<td>−13.1</td>
<td>30°C, pH 7.6</td>
<td>(39)</td>
</tr>
<tr>
<td><em>Synbranchus marmoratus</em></td>
<td>Buccopharyngeal cavity</td>
<td>7.0</td>
<td>−0.69</td>
<td>ND</td>
<td>30°C, pH 7.8</td>
<td>(20)</td>
</tr>
<tr>
<td><em>Channa maculate</em></td>
<td>Suprabranchial organ</td>
<td>7.6</td>
<td>−0.70</td>
<td>ND</td>
<td>25°C, pH 7.6</td>
<td>(69)</td>
</tr>
<tr>
<td><em>Protopterus aethiopicus</em></td>
<td>Lung</td>
<td>10.0</td>
<td>−0.47</td>
<td>ND</td>
<td>25°C, PCO₂ 6 mmHg</td>
<td>(36)</td>
</tr>
<tr>
<td><em>Lepidosiren paradora</em></td>
<td>Lung</td>
<td>7.1</td>
<td>−0.24</td>
<td>ND</td>
<td>23°C, PCO₂ 6 mmHg</td>
<td>(27)</td>
</tr>
<tr>
<td><em>Neoceratodus fosteri</em></td>
<td>Lung</td>
<td>11.0</td>
<td>−0.62</td>
<td>ND</td>
<td>18°C, PCO₂ 3.5 mmHg</td>
<td>(37)</td>
</tr>
</tbody>
</table>

ND, not determined. * denotes hemolysate.
in temperatures (38), as is also the case in aquatic habitats of the African lungfish, which also has blood with a high temperature sensitivity of blood O2 binding (36). The Australian lungfish, in contrast, experiences large temperature fluctuations and has less temperature-sensitive blood (37). Following this analogy, a high-temperature sensitivity might be a tolerable trait in fishes living in stenothermal environments. While the adaptive significance (if any) of a high-temperature sensitivity remains unclear, it might be simply a thermodynamic consequence of the reduced allosteric effector binding.

Molecular interpretation of temperature effect and high affinity. We demonstrated that the Hb has low sensitivity to ATP above pH 7.4 and to Cl− ions over the whole physiological pH range (Figs. 1 and 4). Both anions would normally stabilize Hb in its low O2-affinity tense state conformation and, thus, lower the Hb-O2 affinity (44). The weak oxygenation-linked anion binding, thus, only decreases O2 affinity slightly below the intrinsic Hb-O2 affinity and, thereby, provides a potent mechanism for increasing blood O2 affinity above normal. A similar adaptation has been observed in two other hypoxia-adapted aquatic vertebrates. Hb of the Andean frog Telmatobius peruvianus, inhabiting mountain lakes above 3,800 m is insensitive to Cl− ions (60, 66), as is the Hb of the hypoxia-tolerant swamp eel (10), resulting in a high blood O2 affinity in both cases. Evolution of Hb with reduced Cl− insensitivity, thus, seems to be a common mechanism for efficiently increasing blood O2 affinity in response to hypoxia.

ATP binds to Hb in the physiological pH range, but only decreases Hb-O2 affinity below a pH of ~7.4 (Figs. 1 and 4). This contrasts to the normal trend, where ATP decreases O2 affinity over a larger pH range (11, 15, 66). During environmental hypoxia, intraerythroid ATP concentration decreases in many ectothermic vertebrates, and this is associated with decreased inhibitory interactions of ATP on Hb, which increases the blood O2 affinity during environmental hypoxia (51, 54, 65, 68). The reduced ability for ATP to decrease O2 affinity in P. hypophthalmus Hb consequently only allows for a small degree of hypoxia-induced increase in blood O2 affinity. ATP is present in RBC of many fishes, including some siluriform fishes, and can exert an effect on Hb-O2 affinity in some species (3, 22, 53, 65). Given the weak binding of ATP to Hb, as well as comparable O2 affinities for blood and Hb at 25°C, we can assume that GTP does not exert significant effects on Hb-O2 affinity.

Hb-O2 binding is exothermic, and normally in vertebrate Hbs, oxygenation is linked to an endothermic release of Cl−, organic phosphates, and protons, offsetting the exothermy of oxygenation (63) and, thereby, reducing the temperature sensitivity of O2 binding. Oxygenation-linked ATP binding is much weaker in P. hypophthalmus compared with other species, as evident in the low ΔHATP (3.08 kcal/mol vs. 9.9–21 kcal/mol reported in other species) and the low reduction in O2 affinity upon ATP addition (25, 62). Thus, the lack of oxygen-binding modulation by both ATP and Cl− combine in P. hypophthalmus blood, leaving the exothermy of O2 binding in Hb intact and imparting a high temperature effect on blood O2 affinity.

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

Perspectives and Significance

This study documents how a lack of Cl− binding and weak ATP binding to Hb is associated with a high blood O2 affinity related to inhabiting freshwater environments that are frequently very hypoxic. The weak anion sensitivity of Hb is found in other hypoxia-adapted aquatic vertebrates and allows for high blood O2 affinity. Weak anion sensitivity of Hb limits the modulation of O2 affinity through changes in RBC organic phosphate concentration and is associated with chronic high O2 affinity. An inevitable consequence is a high-temperature sensitivity of blood due to this lack of oxygenation-linked endothermic release of anionic effectors, and this study documents the highest temperature sensitivity of O2 binding measured in blood of an air-breathing fish. This suggests that at low temperatures, O2 unloading may be compromised by a high O2 affinity, limiting O2 transport, whereas higher temperatures may constrain O2 uptake. Therefore, it will be of interest to conduct future studies on the aerobic performance across temperatures in this species to clarify whether this common hypoxia adaptation is associated with a narrow optimal temperature range, as well as a high optimal temperature for aerobic scope.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.D., T.W., and M.B. conception and design of research; C.D. performed experiments; C.D. analyzed data; C.D., F.B.J., T.W., and M.B. interpreted results of experiments; C.D. and F.B.J. prepared figures; C.D. drafted manuscript; C.D., F.B.J., T.W., and M.B. edited and revised manuscript; C.D., L.M.P., D.T.T.H., F.B.J., T.W., and M.B. approved final version of manuscript.

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