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Short communication

Enhanced oxygen unloading in two marine percomorph teleosts

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ABSTRACT

Teleost fishes are diverse and successful, comprising almost half of all extant vertebrate species. It has been suggested that their success as a group is related, in part, to their unique O_2 transport system, which includes pH-sensitive hemoglobin, a red blood cell β-adrenergic Na^+/H^+ exchanger (RBC β-NHE) that protects red blood cell pH, and plasma accessible carbonic anhydrase which is absent at the gills but present in some tissues, that short-circuits the β-NHE to enhance O_2 unloading during periods of stress. However, direct support for this has only been examined in a few species of salmonids. Here, we expand the knowledge of this system to two warm-water, highly active marine percomorph fish, cobia (*Rachycentron canadum*) and mahi-mahi (*Coryphaena hippurus*). We show evidence for RBC β-NHE activity in both species, and characterize the Hb-O_2 transport system in one of those species, cobia. We found significant RBC swelling following β-adrenergic stimulation in both species, providing evidence for the presence of a rapid, active RBC β-NHE in both cobia and mahi-mahi, with a time-course similar to that of salmonids. We generated oxygen equilibrium curves (OECs) for cobia blood and determined the P_{50}, Hill, and Bohr coefficients, and used these data to model the potential for enhanced O_2 unloading. We determined that there was potential for up to a 61% increase in O_2 unloading associated with RBC β-NHE short-circuiting, assuming a $-0.2\ \Delta pH_{a\text{-}v}$ in the blood. Thus, despite phylogenetic and life history differences between cobia and the salmonids, we found few differences between their Hb-O_2 transport systems, suggesting conservation of this physiological trait across diverse teleost taxa.

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Teleost fishes represent almost half of all extant vertebrate species (Nelson et al., 2016). Their widespread success began with the largest adaptive radiation in vertebrate history, during a time of low atmospheric O_2 in which teleosts flourished while many other organisms perished (Helfman et al., 2009). In recent years, it has been proposed that a remarkable O_2 transport system that is unique to teleosts, enhancing their ability to unload O_2 to their tissues, played an important role in their adaptive radiation (Randall et al., 2014).

Most vertebrates possess hemoglobin (Hb) with a Bohr effect, where a decrease in pH decreases Hb-O_2 affinity (Bohr et al., 1904; Jensen, 2004). Teleost Hbs often exhibit a Root effect, where a reduction in pH also decreases the O_2-carrying capacity of Hb, preventing it from becoming fully saturated (Root, 1931; Scholander and Van Dam, 1954). The Root effect, in conjunction with a localized acidosis, is central to generating the high partial pressures of O_2 (PO_2) required to fill the swim bladder at depth and supply O_2 to the avascular retina in the eye (Scholander and Van Dam, 1954; Wittenberg and Wittenberg, 1974; Damsgaard et al., 2020). However, during a generalized acidosis, such as which occurs following exhaustive exercise, the Root effect can be detrimental to O_2 loading at the gills. Thus, many teleosts that possess Root effect Hb also regulate RBC pH_i during a generalized acidosis to protect Hb-O_2 carrying capacity, via β-adrenergically stimulated Na^+/H^+ exchangers (β-NHE) on the RBC membrane that extrude H^+ into the plasma (Nikinmaa, 1982; Primmett et al., 1986; Berenbrink et al., 2005).

The teleost system for enhanced O_2 unloading is thought to require three main components: 1) pH-sensitive Hb, such as the Root effect, 2) rapid red blood cell (RBC) intracellular pH (pH_i) protection via β-NHE activity, and 3) a heterogenous distribution of plasma-accessible carbonic anhydrase (paCA) that short-circuits RBC pH_i protection (by rapidly converting extruded protons back into CO_2 that re-enters the RBC) at the tissues, but not at the gills (Harter and Brauner, 2017;

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Randall et al., 2014; Rummer et al., 2013; Rummer and Brauner, 2011). Together, during a mild acidosis, such as that associated with stress, these traits further reduce RBC pH during tissue transit, increasing the magnitude of the Bohr effect to drive O_2 to the tissues. In Atlantic salmon, this system may reduce cardiac work by up to 30% during exercise and may be an important characteristic associated with the remarkable aerobic migratory ability of salmonids (Harter et al., 2019; Rummer and Brauner, 2015). The presence of a Root effect and RBC β-NHE have been shown to exist in most teleosts (Berenbrink et al., 2005), and assuming a rapid rate of RBC β-NHE and heterologous distribution of paCA, enhanced O_2 unloading has been proposed to be a general trait in teleosts (Harter and Brauner, 2017; Randall et al., 2014; Rummer et al., 2013). However, the presence of paCA and rapid rate of RBC β-NHE have only been investigated in salmonids to date, and specifically only rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), and Atlantic salmon (*Salmo salar*) (Harter et al., 2019; Rummer et al., 2013; Rummer and Brauner, 2015; Shu et al., 2017). The objective of this study was to investigate the presence or absence of traits associated with this phenomenon in species distantly related to the salmonids, in order to determine whether this trait might be more broadly applicable to teleosts.

Here, we chose to investigate cobia (*Rachycentron canadum*) and mahi-mahi (*Coryphaena hippurus*), sister taxa within the percomorph lineage of teleosts. As distantly related species to salmonids, but with a similar athletic, migratory lifestyle (Schlenker et al., 2021) and high aerobic capacity (Nelson et al., 2017; Stieglitz et al., 2016, 2018), cobia and mahi-mahi provided us with a reference point that was relatively diverged from salmonids, but in which enhanced Hb-O_2 unloading would likely be beneficial. Unlike salmonids, however, tropical/subtropical cobia and mahi-mahi are warm-water marine fishes, and this environmental difference may be reflected in their physiology; at higher temperatures, metabolic activity is increased (Heuer et al., 2021), while dissolved O_2 in the water is decreased, increasing the evolutionary selection pressures on a system that could unload more O_2 to the tissues. In addition, mahi-mahi have been shown previously to possess β-NHE activity (Berenbrink et al., 2005), while nothing is currently known about cobia. We thus predicted that cobia and mahi-mahi would show evidence for this system of enhanced O_2 unloading, supporting previous studies hypothesizing the widespread nature of this system throughout teleosts in general.

Our first goal was to confirm the presence or absence of rapid RBC β-NHE activity in cobia and mahi-mahi by measuring the time course of RBC β-adrenergic cell swelling as a proxy, as has been previously conducted in salmonids (Caldwell et al., 2006; Rummer and Brauner, 2011; Shu et al., 2017). In addition, we also determined basic blood-O_2 transport characteristics in cobia; specifically, we generated oxygen equilibrium curves (OEC) on whole blood at two CO_2 tensions to determine blood pH sensitivity (Bohr coefficient), blood oxygen affinity (P_{50}, the PO_2 at which blood is 50% oxygenated), and the potential for enhanced O_2 unloading, according to Rummer and Brauner (2015) and Shu et al. (2017) and for comparison with other salmonids.

Captive bred cobia (916.4 ± 373 g; mean ± s.d., $n = 6$) and mahi-mahi (408 ± 114 g; mean ± s.d., $n = 6$) were reared and maintained in 5000 L cylindrical tanks supplied with flow-through seawater on a natural photoperiod at approximately 29 °C at the University of Miami Experimental Hatchery, as described previously (Benetti et al., 2008; Stieglitz et al., 2012, 2017). Both species were fed daily to satiation with a combination of chopped squid and sardines as well as commercial marine fish pellets (Skretting, Toole UT). All study procedures were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC protocol #15–019).

Each individual fish was netted out of the main holding tank and rapidly transferred to a 20 L bucket filled with oxygenated and filtered seawater and then anesthetized in tricaine methanesulphonate (MS-222, 0.2 g/L) buffered with $NaHCO_3$ (0.4 g/L) dissolved in seawater. When the fish became unresponsive but was still ventilating, it was removed from the water, then 6 mL of blood was drawn via caudal venipuncture into heparinized syringes. Blood was centrifuged, and RBCs were rinsed twice and re-suspended in marine teleost saline (in mM: 150 NaCl, 5.4 KCl, 1.5 MgCl2, 3.2 CaCl2, 10 glucose, and 10 HEPES, at pH 7.7; Brette et al., 2014) and stored at 4 °C overnight to allow any catecholamines to degrade (Caldwell et al., 2006).

Rinsed and resuspended RBCs from each individual fish were divided into two 2 mL aliquots and placed in rotating Eschweiler tonometers. Samples were allowed to equilibrate at 29 °C to a humidified custom-mixed gas (5% O_2 and 1% CO_2, balanced with N_2) to generate hypoxic and acidified blood to potentiate the RBC β-NHE response (Caldwell et al., 2006). Blood was equilibrated to these conditions for at least 30 min before Hct was measured and then standardized to 25% by adding marine teleost saline or removing plasma. Samples were drawn to measure pH_e, Hct, and [Hb] (time 0). Then isoproterenol (ISO) was added to a final concentration of 10^{-5} M ISO (final concentration in blood; Sigma-Aldrich, I5627; Rummer and Brauner, 2011), and samples were drawn at 5, 15, 30, and 60 min, with unstimulated duplicate tonometers as paired controls for measurement of Hct and [Hb], as per Shu et al. (2017) and calculation of mean corpuscular hemoglobin concentration (MCHC; [Hb]/Hct x 100). As an additional control, the experiment was repeated, as described above, in cobia blood, but with propranolol (a β-NHE blocker) that was added 5 min before ISO to confirm that cell swelling was due to a β-adrenergic effect. To determine RBC β-NHE activity (Nikinmaa, 1992), the effect of time on Hct in the presence and absence of ISO was analysed using a repeated measures ANOVA, followed by a post-hoc Dunnett's test. The total change in MCHC (ΔMCHC) was calculated between 0 min and 60 min in the presence and absence of ISO.

In cobia blood, Hct was found to increase significantly 5 min following stimulation with ISO (25.2 ± 0.1% at 0 min to 26.2 ± 0.3% at 5 min; $p < 0.0001$; Fig. 1A). An increase in Hct continued over the course of the experiment to a final Hct of 27.8 ± 0.4% at 60 min. Similarly, in mahi-mahi blood, Hct also increased significantly by 5 min (25.1 ± 0.3% at 0 min to 26.5 ± 0.4% at 5 min; $p < 0.001$; Fig. 1A), and reached a final Hct of 27.8 ± 0.5% at 60 min. The Hct for both control (unstimulated) and propranolol-treated samples did not differ significantly from 0 min at any time point for either species. At 60 min, the final Hct values for both ISO-stimulated cobia and mahi-mahi blood were significantly different from the unstimulated and propranolol control values ($p < 0.0001$; Fig. 1A). In a similar manner, MCHC in ISO-stimulated blood decreased by 0.40 ± 0.07 mmol/L for cobia and by 0.34 ± 0.06 mmol/L for mahi-mahi. Unstimulated and propranolol-treated blood changed by 0.19 ± 0.09 and 0.01 ± 0.06 mmol/L for cobia blood and by 0.01 ± 0.06 mmol/L for unstimulated mahi-mahi blood, respectively. Blood pH was approximately 7.16 ± 0.02 for cobia and 7.08 ± 0.04 for mahi-mahi during these experiments.

Following RBC β-adrenergic stimulation, both cobia and mahi-mahi blood Hct values increased and MCHC values decreased significantly within 5 min. This was a response similar to coho salmon, more rapid than in rainbow trout, but not as rapid as Atlantic salmon (Shu et al., 2017). Final differences in Hct in cobia and mahi-mahi between control and stimulated blood were also comparable to coho salmon and rainbow trout, with a 12.1% and 9.5% increase in cell volume in cobia and mahi-mahi blood respectively (24.8 Hct to 27.8 for cobia, 25.4 Hct to 27.8 for mahi), and a 9.2% and 11.4% increase in rainbow and coho trout cell volume (24.7 Hct to 27 for rainbow trout, 24.9 Hct to 27.8 for coho; Shu et al., 2017). Based on these data, we conclude that both cobia and mahi-mahi do, in fact, possess a rapid RBC β-NHE response.

To characterize blood Hb-O_2 transport in cobia, OECs were generated. Blood was collected as described above from 6 mahi-mahi and then shipped overnight on ice in EDTA-coated 3 mL BD Vacutainer tubes to the Department of Zoology, University of British Columbia (UBC). Upon arrival, blood from each fish was pooled into heparinized 10 mL Vacutainer tubes and maintained on ice. Over the next 72 h, blood samples were stored at 4 °C, and OECs were generated using a parallel assay of

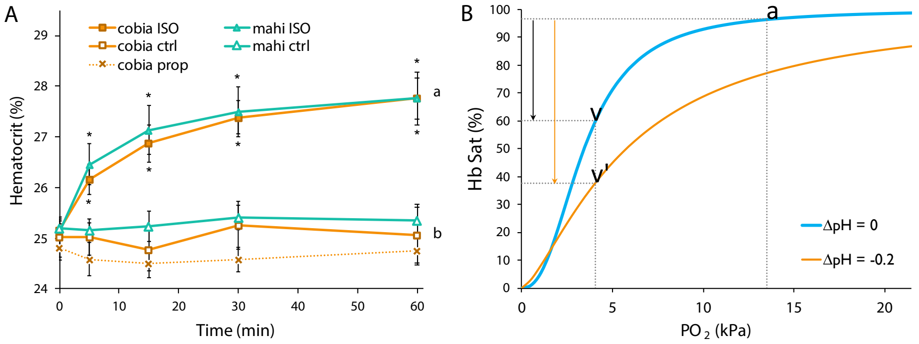

Fig. 1. A) The red blood cell (RBC) β-adrenergic cell swelling response in resuspended RBCs from cobia (*Rachycentron canadum*) and mahi-mahi (*Coryphaena hippurus*). RBCs were stimulated with isoproterenol (ISO; 10^{-5} M), unstimulated (ctrl), or blocked with propranolol (prop) before stimulating with ISO, and hematocrit was monitored over 60 min. Asterisks (*) indicate statistically significant differences from 0 min, and different letters indicate differences in hematocrit at 60 min between treatments and controls. B) Oxygen equilibrium curves (OECs) of cobia (*R. canadum*) whole blood. Curves were calculated from measured mean P_{50} and n_H values determined at PCO_2 levels of 0.25 and 1 kPa, representing Hb-O_2 binding at resting pH and at a ΔpH of −0.2. **a** indicates the Hb-O_2 saturation at an arterial partial pressure of oxygen (P_aO_2) of 13.5 kPa, while **v** and **v'** demarcate the Hb-O_2 saturation at a venous partial pressure of oxygen (P_vO_2) of 4.1 kPa at each pH. These P_aO_2 and P_vO_2 values correspond with in vivo arterial and venous PO_2 found in routinely swimming rainbow trout (Brauner et al., 2000); such measurements have not yet been made in cobia. Arrows indicate the amount of O_2 unloaded at each pH. $n = 6$ for each trace. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Hematological parameters for cobia (*R. canadum*) whole blood.

Parameter	0.25 kPa CO_2	1 kPa CO_2
pH_e at P_{50}	7.72 ± 0.07^a	7.52 ± 0.03^b
P_{50} (Torr)	25.72 ± 1.99^a	43.27 ± 2.43^b
Hill coeff. (n_H)	2.41 ± 0.41^a	1.42 ± 0.13^a
Bohr coefficient	-0.6 ± 0.21	
Hct (%)	39.7 ± 3.09	
[Hb] (mmol/L)	1.47 ± 0.04	
MCHC (mmol/L)	3.83 ± 0.33	

Blood was sampled caudally and used to produce oxygen equilibrium curves at two PCO_2 levels.
Letters that differ within each row denote statistically significant differences; $n = 6$.
Hct, hematocrit; [Hb], hemoglobin concentration; MCHC, mean cell hemoglobin concentration.
Hct, [Hb], and MCHC represent mean ± S.E.M. from all individuals at both PCO_2 levels.

oxygen equilibria as described by Lilly et al. (2013) and Shu et al. (2017) using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). To do this, samples were loaded between two sheets of polyethylene film (a thin, O_2-permeable membrane), secured in place with two O-rings on a microcuvette, then placed in a multi-cuvette tonometer that positioned the samples for spectrophotometric analysis in the microplate reader. A Wösthoff gas mixing pump (Bochum, Germany) was used to produce, humidify, and pump mixtures of N_2, O_2, and CO_2 over the microcuvettes while absorbance of blood samples was measured in parallel at 430 nm and 390 nm. The OECs were determined for each species at 0.25 and 1 kPa CO_2 by measuring absorbance after blood had equilibrated with stepwise increases of O_2, as performed using salmonid blood in Shu et al. (2017). All OEC analyses were conducted at 29 °C. Values of pH for parallel blood samples at each PCO_2 were determined via tonometry at 29 °C with custom-mixed gases, according to Shu et al. (2017).

Fractional hemoglobin saturation (S) at a specific PO_2 was calculated as in Shu et al. (2017), then plotted against incubation PO_2. A curve was fitted to the points using a three-parameter logistic equation. Using this model, OECs were generated and P_{50} values were calculated for each fish at each PCO_2. A second-degree polynomial was used to correlate S with pH measurements, and pH at P_{50} (pH_{50}) was interpolated based on the estimated parameters.

Representative OECs were reconstructed using the Hill equation from mean P_{50} and n_H values determined at each PCO_2. The Bohr coefficient was calculated by taking the slope of a regression line fitted to all points of $\log P_{50}$ vs. pH via a linear mixed-effects model for each PCO_2 level (Weber et al., 1976). Then, pH, P_{50}, and Hill coefficients were compared between PCO_2 levels using one-way ANOVAs with $\alpha = 0.05$.

With evidence of a rapid, active RBC β-NHE, the potential for enhanced O_2 unloading was modelled using the cobia OECs generated here, assuming a fixed arterial-venous PO_2 difference ($\Delta P_{a\text{-}v}O_2$) at two pH levels, as described in previous studies (Rummer et al., 2013; Rummer and Brauner, 2015; Shu et al., 2017). A P_aO_2 of 13.5 kPa (**a** in Fig. 1B) and a P_vO_2 of 4 kPa (**v** in Fig. 1B) were used, corresponding with in vivo arterial and venous PO_2 values, respectively, measured in routinely swimming rainbow trout (Brauner et al., 2000). These values were used because no in vivo PO_2 measurements have been previously made in cobia. In the absence of a change in blood pH, O_2 unloading (ΔS) was calculated as the difference in saturation (S) at the $\Delta P_{a\text{-}v}O_2$ for the OEC conducted at 0.25 kPa CO_2. This was found to be 36.5% (Fig. 1B, change from **a** to **v**, black arrow). Assuming a blood acidosis of 0.2 pH units (i.e., reduction in blood pH from 7.72 to 7.52; Table 1) that would be associated with a moderate stressor, the potential benefit of RBC β-NHE short circuiting to O_2 unloading was calculated assuming the same arterial saturation (Fig. 1B, point **a**) and $\Delta P_{a\text{-}v}O_2$, but with the venous saturation that would result from a right-shift of the OEC (Fig. 1B, change from **a** to **v'**, orange arrow). This ΔS was found to be 58.8%, corresponding to a 61% increase in O_2 unloading (36.5 to 58.8%). Thus, assuming a mild acidosis in the blood of 0.2 pH units, RBC β-NHE short-circuiting at the tissues and recovery at the gills could conceivably increase O_2 unloading by 61%. This value is comparable to what has been found in salmonids previously using a similar analysis (27%, 76%, and 62% increase in Atlantic salmon, coho salmon, and rainbow trout, respectively; Shu et al., 2017), however whether this would occur in vivo remains to be investigated.

The purpose of this study was to investigate the potential for enhanced O_2 unloading associated with RBC β-NHE short-circuiting in a distantly related, non-salmonid teleost. Here, we show evidence for the presence of this system in cobia and mahi-mahi. Our results indicate that the blood of cobia and mahi-mahi, both species that are highly active and that live in warm waters (Nelson et al., 2017; Stieglitz et al., 2016, 2018), exhibit rapid and pronounced RBC β-NHE activity. In cobia blood, we found a high pH sensitivity, with a large Bohr coefficient and Root effect. This satisfies 2 of the 3 requirements for enhanced O_2 unloading associated with RBC β-NHE short-circuiting; the third requirement, a heterogenous distribution of paCA at the tissues but not at the gills, has not yet been investigated in either of these species, but is likely given the presence of RBC β-NHE activity (Randall et al., 2014; Harter and Brauner, 2017). From these data, we can conclude that there is at least a large potential for enhanced O_2 unloading in these fish associated with RBC β-NHE short-circuiting (that needs to be validated in vivo), and that, despite phylogenetic and environmental differences between cobia and salmonids, the dynamics of β-NHE activity and quantitative estimates of enhanced O_2 unloading appear to be comparable. This system for enhanced O_2 unloading has been shown to be important in salmonids during exercise in Atlantic salmon (Harter et al., 2019), in ensuring O_2 delivery to the spongy myocardium in the heart of coho salmon (Alderman et al., 2016), and in O_2 secretion to the avascular eye of rainbow trout (Damsgaard et al., 2020). Given that cobia and mahi-mahi diverged from the salmonids more than 200 mya (Betancur-R et al., 2013) but also appear to possess the traits for enhanced O_2 unloading (Randall et al., 2014; Harter and Brauner, 2017), this, along with that of Berenbrink et al., 2005, provides support for the hypothesis that not only salmonids, but teleosts in general, or at least the euteleosts, may be able to take advantage of this system. However, given the diversity of teleosts and their wide range of lifestyles and habitats, we have only begun to scratch the surface of investigating this system; clearly, this is an area worthy of further research.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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