



Very high blood oxygen affinity and large Bohr shift differentiates the air-breathing siamese fighting fish (*Betta splendens*) from the closely related anabantoid the blue gourami (*Trichopodus trichopterus*)

J.F. Mendez-Sanchez^a, W.W. Burggren^{b,*}

^a Departamento de Biología, Universidad Autónoma del Estado de México, Mexico

^b Department of Biological Sciences, University of North Texas, Denton, TX, USA

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ABSTRACT

The Siamese fighting fish, *Betta splendens*, and the blue gourami, *Trichopodus trichopterus*, are two closely related air-breathing anabantoid fishes. *B. splendens* is a sedentary facultative air breather frequenting often hypoxic waters, while *T. trichopterus* is a more active obligatory air-breather inhabiting better oxygenated waters. Despite their close taxonomic relationship, previous studies have shown inter-specific differences in both physiological and morphological plasticity. Consequently, we hypothesized that *B. splendens* would have the higher blood oxygen affinity characteristics typical of more hypoxia-tolerant fishes. Whole blood oxygen equilibrium curves were determined at 27 °C and pHs of 7.62, 7.44 and 7.25. At a pH of 7.62, the blood O₂ affinity (P₅₀) of *B. splendens* was just 2.9 mmHg, while that of *T. trichopterus* was ~5 times higher at 14.7 mmHg. There were no significant differences in P₅₀ between males and females in either species. The Bohr coefficient in *B. splendens* and *T. trichopterus* was -1.79 and -0.83, respectively. *B. splendens*, unlike *T. trichopterus*, showed a large Root effect. Hills cooperatively coefficient, *n*, was ~2 in both species, indicating a significant binding cooperative between oxygen and hemoglobin. Collectively, these differences in blood O₂ transport characteristics in these two closely related species are likely correlated with the differing habitats in which they breed and inhabit as adults, as well as different activity levels. Finally, the very high blood O₂ affinity of *B. splendens* is not extraordinary among air-breathing fish, as revealed by a review of the literature of blood oxygen affinity in air-breathing fishes.

1. Introduction

The blue gourami, *Trichopodus trichopterus* (formerly *Trichogaster trichopterus*), and the Siamese fighting fish, *Betta splendens*, are two closely related anabantoids that breathe air with their highly derived labyrinth organ (Tate et al., 2017). Despite this taxonomic similarity, as larvae and juveniles, at least, these two species show quite different morphological, behavioral and physiological responses to environmental changes, especially in ambient oxygen levels. For example, *B. splendens* is a sedentary, facultative air-breather (uses but does not require air breathing), while *T. trichopterus* is a more active obligate air-breather. Larvae of both species under continuous hypoxia showed only a 15% survival rate. *B. splendens* respond to hypoxic rearing with increased hypoxic tolerance and a 4 day advance in the onset of air breathing, whereas larval *T. trichopterus* showed few physiological responses to chronic hypoxia, reflecting a lower degree of developmental

phenotypic plasticity (Mendez-Sanchez and Burggren, 2014 and 2017). Larval and juvenile *B. splendens* also respond with quite different morphological adjustments to rearing in chronic hypoxia, showing larger adjustments in respiratory surface area of the gills and air-breathing labyrinth organ compared to *T. trichopterus* where the adjustments are rather minor (Mendez-Sanchez and Burggren, 2019). These differences likely relate to differences in the nature of the environments occupied by the two species. Thus, *B. splendens* will invade temporary ponds that can experience severe nocturnal hypoxia, whereas *T. trichopterus* tends to inhabit more permanent well-oxygenated bodies of water (Rainboth, 1996; Monvises et al., 2009). Additionally, differences between species exist in air-breathing behavior - facultative vs obligate (Graham, 1997; Lefevre et al., 2014; Mendez-Sanchez and Burggren, 2014).

Increases in blood oxygen affinity (measured as P₅₀, the PO₂ at which blood is 50% oxygen saturated) and decreases in blood affinity

* Corresponding author.

E-mail addresses: fms@uaemex.mx (J.F. Mendez-Sanchez), burggren@unt.edu (W.W. Burggren).

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caused by decreasing pH (Bohr shift) are additional important mechanisms for coping with environmental hypoxia, even in air-breathing fishes that can partially escape the consequences of aquatic hypoxia by breathing air (Mandic et al., 2009; Richards, 2011; Martin, 2014). Consequently, blood oxygen transport phenotype has been heavily selected for in air-breathing fishes (Morris and Bridges, 1994; Graham, 1997). However, blood oxygen affinity, Bohr shift and Root effect (the inability of blood to become fully oxygen saturated at even high PO_2 s as a result of decreasing blood Ph) have not been compared in *B. splendens* or *T. trichopterus*. Whether these blood variables continue the trend of showing marked differences between these species based on their respective habitats is unknown. Moreover, there is scant information on blood characteristics of anabantoid fish generally, doubtlessly because of the small size and blood volumes of many of these anabantoid species (Zanjani et al., 1969). A brief report the blood oxygen affinity characteristics of the blue gourami, indicating that *T. trichopterus* has a “low oxygen affinity” at ~ 17 mmHg (pH 7.4, 28 °C) and a “relatively low” Bohr shift of -0.35 (Herbert and Wells, 2001). However, the existing data on blood characteristics typically comprise blood hematology values rather than variables associated with blood gas transport physiology.

Against this backdrop, we hypothesize that the differences in habitat and lifestyle and thus respiratory biology between *Betta splendens* and *Trichopodus trichopterus* will by extension also apply to the blood oxygen transport properties of these two species. Specifically, we predict that a higher oxygen affinity and greater Bohr shift – attributes generally regarding as improving oxygen transport in conditions of aquatic hypoxia – will occur in *B. splendens* when compared to *T. trichopterus*. We additionally hypothesized that blood oxygenation characteristics differ between males and females (a rarely tested factor in the literature on fish blood characteristics).

2. Methods

2.1. Fish Stock

Ten adult *Trichopodus trichopterus* (5.50 ± 0.50 g) and ten adult *Betta splendens* (1.35 ± 0.15 g), comprising 5 females and 5 males for each species, were obtained from breeding stock in the Department of Biological Sciences at the University of North Texas, Denton, Texas, USA. These fish were maintained in fully air-saturated water at 28 °C, under a 12 h light:12 dark lighting regime. Fish were fed twice daily with commercial flake fish food. All experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee of the University of North Texas.

2.2. Blood sampling

Blood was sampled by cardiac puncture (e.g. Zanjani et al., 1969; Ostrander, 2000). Individual fish were first anesthetized using a buffered solution of MS222 (1:1000 in aquarium water). Immediately upon losing equilibrium, the fish was removed from water and immediately positioned ventral side up in a holding cradle. A 1 ml glass syringe that had been pre-wetted with 1000 U/ml heparin (Chantler and Cox, 1999) was fitted with a 22-gauge needle, the tip of which was inserted into the ventricle directly through the skin and bone into the area of the ventral aorta. A 50 μ l blood sample, representing an estimated ~ 3 –4% of blood volume for *B. splendens* and 0.8–1% for *T. trichopterus*, was then carefully withdrawn, avoiding unnecessary suction that might cause red blood cell lysis. Samples yielding insufficient blood were discarded, as also were blood samples requiring > 30 s to withdraw after fish lost equilibrium. Preliminary study of sampled blood under a dissecting microscope revealed no evidence of hemolysis.

After blood collection, each fish was euthanized by returning it to the buffered MS222 solution until all opercular movement stopped.

2.3. Hemoglobin-oxygen equilibrium curves

Fifty microliters of freshly drawn blood from one fish individual were mixed with 5.0 ml of a proprietary solution of HEMOX-Buffer Solution, 20 μ l of HEMOX Additive-A, and 10 μ l of Anti-Foaming Agent (TCSSCI Scientific Supplies). The dilute whole blood solution (5.06 ml in total) was immediately placed in an automated blood oxygen dissociation analyzer (Hemox Analyzer Model B, TCS Scientific Corp) maintained at 27 °C with a zero calibration performed prior to each run using sodium sulphite solution. Essentially, the Hemox system is a spectrophotometer that monitors wavelength absorbance in the band relevant to oxy- and deoxy-hemoglobin, while dynamically altering the PO_2 of the dilute blood solution using proscribed gas mixtures of nitrogen and air containing fixed amounts of carbon dioxide (to fix blood pH at a specified level). The blood oxygen equilibrium curve (OEC) was generated over a 40–60 min period of time, starting from completely oxygen desaturated blood. Assessments of the effect of pH on blood oxygen equilibrium curves were determined by adjusting pH in the HEMOX buffer-blood solution to 7.62 ± 0.02 (0.0% CO_2), 7.44 ± 0.02 (1.0% CO_2) and 7.25 ± 0.03 (1.0% CO_2) Gas mixtures for the HEMOX analyzer were produced with Wösthoff gas mixing pumps. These relationships at 27 °C between HEMOX buffer pH and each equilibration CO_2 was separately determined in triplicate using a Wösthoff gas mixing pump to produce a precise CO_2 mixture ($\pm 0.1\%$) in a 5.0 ml of HEMOX solution while measuring pH with a Radiometer ABL 5 system pH electrode.

Hemoglobin oxygen affinity of whole blood was measured as P_{50} , the partial pressure in mmHg at which the blood was 50% saturated with oxygen, as determined from each completed OEC.

Hill's n , expressing the sigmoidicity of the curve and a reflection of the cooperativity between oxygen and the hemoglobin molecule, was calculated from the slope of a line in a plot of: $\log(O_2 \text{ saturation}/100 - O_2 \text{ saturation})$ plotted against $\log PO_2$, over the range 20% to 70% O_2 saturation. A minimum of 10 data points within this range were employed to create the slope of the line.

The Bohr effect, Φ , was determined from the equation $\Phi = \Delta \log P_{50}/\Delta pH$ and was estimated for each experimental individual over the pH intervals 7.62 to 7.44, 7.44 to 7.25 and 7.62–7.25.

The Root effect, the decrease in maximal oxygen saturation at air saturation levels caused by decreased pH, was determined as the % decrease in maximal oxygen saturation. The extent of the Root effect differed with different pH ranges, so this value is reported for each of the three pH values at which OECs were created.

2.4. Statistics

Statistical tests of differences in whole blood P_{50} between sexes within species using Kruskal Wallis (H) and U of Mann-Whitney (W) non-parametric analyses. P_{50} and maximum oxygen saturation of hemoglobin-oxygen at different pHs were compared within species with an ANOVA or the equivalent nonparametric Kruskal-Wallis. Hill's n values were compared within species with a multiple linear regression comparison of slopes. Differences in the Bohr effect over different pH intervals were tested with ANOVAs. Differences between species for each blood variable were tested with a t -test. All values are reported as means \pm standard error unless otherwise indicated.

3. Results

3.1. Blood oxygen-hemoglobin affinity: effect of sex

Differences in general body morphology between males and females of *T. trichopterus* are characterized primarily by a longer, pointed dorsal fin in males. However, *B. splendens* exhibits major sexual dimorphism, with females generally smaller and lacking the elaborate dorsal, pelvic and tail fins exclusively to the larger males. Consequently, effects of sex

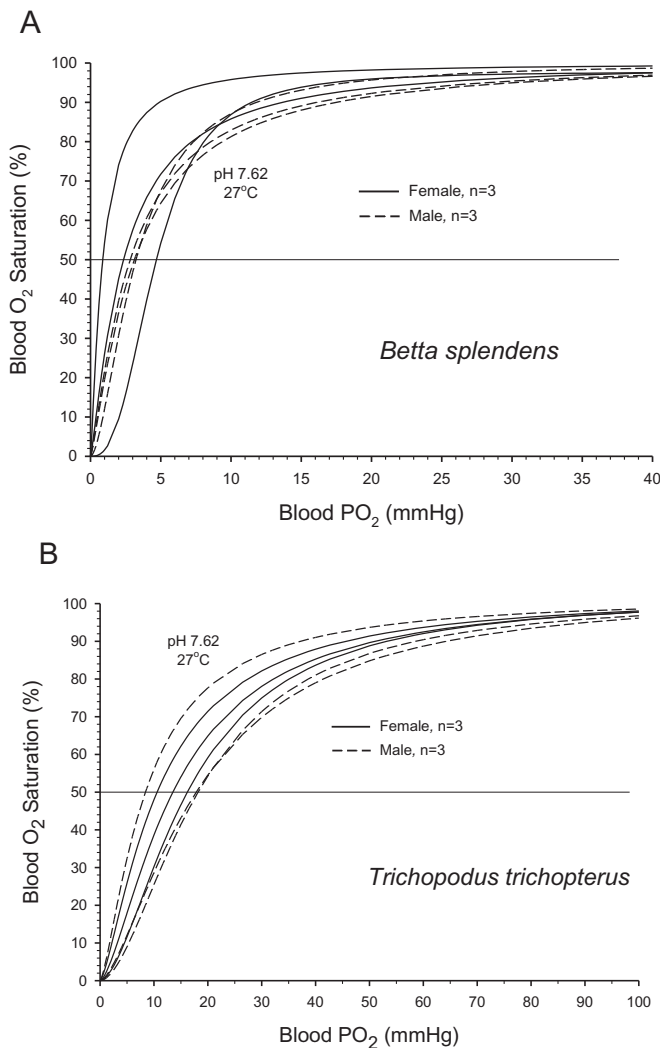


Fig. 1. Oxygen equilibrium curves of whole blood for three males and three females of A) Siamese fighting fish, *Betta splendens* and B) the blue gourami *Trichopodus trichopterus*. Each curve was created at a pH of 7.62 and temperature of 27 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the characteristics of whole blood were first assessed. Blood was successfully drawn from three adult male and three adult female *B. splendens*, and was used to create individual blood OECs at 27 °C and pH 7.62 (Fig. 1A). There was no significant difference between males and females in blood oxygen affinity at O₂ saturation levels of 50%, 80% or 90% ($n = 3$ at each sex and saturation level, $P > .05$ Kruskal Wallis test). For *T. trichopterus*, just as for *B. splendens*, 3 females and 3 males were sampled, and again there were no significant differences between whole blood oxygen affinity at 27 °C and pH 7.62 at 50%, 80% and 90% saturation ($n = 3$ at each sex and saturation level, $P > .05$ Kruskal Wallis test) (Fig. 1B).

Given no statistical differences between the OECs of males and females within a species, sex was not used as a factor in measuring blood oxygen affinity data in subsequent analyses.

3.2. Species comparison of blood oxygen affinity

Although closely related anabantoid species, there are highly significant ($P < .01$) differences between the blood-oxygen affinity of *B. splendens* and of *T. trichopterus* (Table 1). Whole blood of *B. splendens* exhibits a very high oxygen affinity, with an average P_{50} of just

2.9 ± 1.3 mmHg at pH 7.62 and 27 °C (Fig. 2, Table 1). In contrast, *T. trichopterus* had a P_{50} under identical temperature and pH of 14.2 ± 4.4 mmHg, ~5 times higher than *B. splendens* (W (Coefficient of Concordance for ranked data) = 36; $n_1 = 6$, $n_2 = 6$; $p < .01$). $n_1 = 6$ *B. splendens* individuals and $n_2 = 6$ *T. trichopterus* individuals, with data from three males and three males combined for each species.

3.3. Effect of blood pH: Bohr shift

Whole blood oxygen-hemoglobin equilibrium curves at pHs 7.62, 7.44 and 7.25 are indicated for both species in Fig. 3. The Bohr coefficient in *B. splendens* over the entire test range of pH 7.62 to 7.25 was -1.79 , indicating a high sensitivity to protons (Table 1). *B. splendens* showed a much larger Bohr effect over the pH interval 7.62 to 7.44 compared to the larger pH interval of 7.44 to 7.25. The Bohr shift for *T. trichopterus* over the entire test range of pH 7.62 to 7.25 was -0.83 , significantly lower ($P < .0001$) than in *B. splendens* (Table 1).

3.4. Hill's cooperativity coefficient

Hill's n for *B. splendens* and for *T. trichopterus* at all three test pHs are provided in Table 1. The value of n was a function of the pH at which this value was determined, with n increasing with decreasing pH. The value of n for both species, ≥ 2.2 , suggests significant binding cooperativity between oxygen and hemoglobin. Generally, n was higher in *B. splendens* than in *T. trichopterus* (Table 1).

3.5. Root effect

In *B. splendens*, blood at pH 7.62 and 27 °C was essentially fully oxygen saturated at a $PO_2 \sim 150$ mmHg (Fig. 3A). However, decreasing blood pH significantly reduced maximum blood O₂ saturation at air saturation (the so-called Root effect) ($F = 29.93$; $df = 2,9$; $p < .001$) (Table 1). In fact, whole blood at a PO_2 of ~ 150 mmHg and pH 7.25 was only 84% O₂ saturated (Table 1), indicating a sizeable Root effect in *B. splendens*.

In *T. trichopterus* there were two individuals showing a depression of blood oxygenation at the lowest test pH of 7.25, but the remaining four did not. This led to large variation about the mean, and thus no significant decrease in maximum blood O₂ saturation caused by decreased blood pH in *T. trichopterus* ($F = 1.6$; $df = 2,11$; $p > .05$) (Table 1, Fig. 3B).

4. Discussion

4.1. Species comparisons of blood oxygen transport properties

4.1.1. Lack of blood sexual dimorphism in blood characteristics

Previous studies have investigated general hematological variables as a function of sex in air-breathing fishes. For example, there are no differences between males and females in blood hematology in the air-breathing reedfish, *Erpetoichthys calabaricus* (Beitinger et al., 1985) and the mud eel *Amphipnous cuchia* (Mishra et al., 1977). To our knowledge, however, differences in blood oxygenation properties (e.g. P_{50} , Bohr shift) have not been examined as a function of sex in air-breathing fishes (and few marine or aquatic species). In fact, failure to distinguish sexes could contribute in part to the variation in comparative physiological data (Burggren, 2014). In defense of past researchers, however, the natural history and morphology of many species is poorly understood, and it may not even be possible to visually distinguish male from female. Fortunately, sexual dimorphism is apparent in anabantoids, especially *B. splendens* allowing us to determine that there are no differences in blood oxygen affinity and as a consequence pool blood data.

4.1.2. Blood oxygen affinity

We hypothesized that *B. splendens* would have a lower P_{50} (and

Table 1
Blood oxygen equilibrium curve variables determined on whole blood (27 °C) of *Betta splendens* and *Trichopodus trichopterus*.

| Blood variable | Ph value or Ph interval | species | | Species differences |
|---|-------------------------|---------------------------|----------------------------------|---------------------|
| | | <i>Betta splendens</i> | <i>Trichogaster trichopterus</i> | |
| Whole Blood P ₅₀ (mmHg) | 7.62 | 2.9 ± 0.3 ^a | 10.5 ± 1.4 ^A | P < .001 |
| | 7.44 | 10.5 ± 0.1 ^b | 14.2 ± 1.1 ^B | P < .0001 |
| | 7.25 | 15.8 ± 0.2 ^c | 25.2 ± 1.2 ^C | P < .0001 |
| Bohr Effect, Φ | 7.62↔7.25 | -1.79 ± 0.04 ^a | -0.83 ± 0.18 ^A | P < .0001 |
| | 7.62↔7.44 | -3.97 ± 0.17 ^b | -0.78 ± 0.15 ^A | P < .0001 |
| | 7.44↔7.25 | -1.00 ± 0.11 ^c | -1.32 ± 0.14 ^B | P < .03 |
| Hills Cooperativity Coefficient, n | 7.62 | 2.2 ± 0.1 ^a | 2.2 ± 0.1 ^A | NS |
| | 7.44 | 3.3 ± 0.1 ^b | 2.4 ± 0.1 ^{A,B} | P < .05 |
| | 7.25 | 4.4 ± 0.2 ^c | 2.7 ± 0.1 ^B | P < .05 |
| Root Effect (% O ₂ Saturation at PO ₂ ~ 150 mmHg) | 7.62 | 100.0 ± 0.4% ^a | 99.8 ± 0.1% ^A | NS |
| | 7.44 | 91.9 ± 1.0% ^b | 98.4 ± 1.2% ^A | P < .002 |
| | 7.25 | 84.3 ± 4.1% ^c | 93.8 ± 6.3% ^A | NS |

Blood oxygen equilibrium curves were performed at one of three pHs, maintained by buffer solutions equilibrated to fixed CO₂ levels (see Methods). Means ± 1 standard error are presented. *n* = 6 for each species. Lower case letters represent statistical similarities/differences within *B. splendens*, while upper case letters make comparisons within *T. trichopterus*, at *P* < 0.05 or lower. The right hand-most column shows statistical significance, if any, between the two species for each blood variable. NS, not significant.

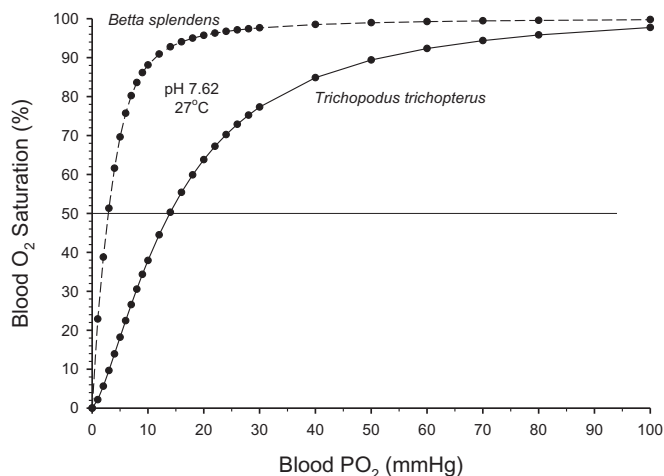


Fig. 2. A comparison of the oxygen-hemoglobin equilibrium curves of whole blood for the blue gourami and the Siamese fighting fish, *Betta splendens* generated under identical conditions of pH 7.62 and 27 °C. Each curve is a composite generated from a model based on individual curves from six different individuals of each species, from Figs. 1 and 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

greater Bohr shift) than *T. trichopterus*, based on behavioral, morphological and physiological differences. This hypothesis was supported by our data. The whole blood of the blue gourami, *T. trichopterus*, has a P₅₀ value of 10.5 mmHg at 27 °C and a pH of 7.62 (Table 1). P₅₀ value for this species was reported to be 17 mmHg at a pH of 7.4 and 28 °C (Herbert and Wells, 2001), compared to a value in the present study of 14.2 at pH 7.44. The values for *T. trichopterus* from the present study are also well within the range of P₅₀ values reported for numerous other air-breathing fishes, with data for > 40 species now published (see Graham, 1997 and additional references in Table 2).

In contrast to the blue gourami, the whole blood of *B. splendens* at 27 °C and a pH of 7.62 exhibits a blood oxygen affinity of just ~3 mmHg, one-fifth that of the blue gourami. While the Siamese fighting fish has a very high blood oxygen affinity compared to almost other Teleosts, it is actually within the range of a small but significant subset of air-breathing fishes (Table 2), as will be discussed below.

4.1.3. Bohr shift

The Bohr shift in both *B. splendens* and *T. trichopterus* is large

compared to many other fishes. An analysis of Φ in water-breathing fishes revealed values for marine fishes are between -0.9 and -1.06, and for freshwater fish species -0.6 and -0.98 Herbert et al. (2006). Among air-breathing fish with high O₂ affinity hemoglobins, Bohr effects range from being nearly proton insensitive (Φ = -0.02 in *Pterygoplichthys anisitsi*, da Cruz et al., 2013; 0 in *Arius leptaspis*, Wells et al., 2005) to values of nearly -2 (Φ = -1.8 in *Amia calva*, Johansen et al., 1970). Thus, the Bohr shift of -1.26 over the pH range 7.62–2.25 for *T. trichopterus* is towards the higher end for all fishes but unremarkable given the variation in Bohr shift of air-breathing fish. The Bohr shift of *B. splendens*, on the other hand, is the highest reported to date for air-breathing fishes. Whether the overall high but different values of the Bohr shift is related to differences in the habitats and behavior of this species, or is a characteristic of all anabantoids, remains to be determined.

4.1.4. Hill's cooperativity coefficient

Hill's *n* will approximate 1 where the oxygen equilibrium curve is hyperbolic and hemoglobin exhibits low cooperativity with oxygen. When hemoglobin shows more cooperativity, *n* can increase to 3.0 for a strongly sigmoidal curve, and fish typically have *n* values between 1.0 and 2.0 (Eddy, 1973; Wells, 2009; Mandic et al., 2009). Both *T. trichogaster* and *B. splendens* exhibit a Hill's coefficient value of ~ 2.2 at pH 7.62 (Table 1), suggesting moderate cooperativity between hemoglobin and oxygen. This value compares favorably with *n* values of 2.0–2.8 for 13 air-breathing sculpins and for air-breathing fishes with high oxygen affinity blood (Table 2).

High values of Hill's cooperativity coefficient have been suggested to be an evolutionary adaptation to hypoxia tolerance in intertidal sculpins (Scorpaeniformes: Cottidae) (Mandic et al., 2009), in the marine fishes *Pagrus auratus* (Cook et al., 2013) and *Pleuronectes platessa* (Wood et al., 1975), and in some freshwater teleosts (Berenbrink et al., 2005). Interestingly, Hill's *n* values of > 4.0 were recorded in very acidic blood of *B. splendens*, suggesting tetramer-tetramer interactions. This so-called 'super-cooperativity' is more common than might be suspected, having been identified in bird and mammal embryos, and in the adults of fish, amphibians, reptiles, birds and marsupials, where it is proposed to aid oxygen unloading over narrow oxygen partial pressure ranges (Knapp et al., 1999; also Grispo et al., 2012).

4.1.5. Root effect

Lowered pH of whole blood in Teleost fishes often (but not necessarily) decreases the maximum possible oxygen hemoglobin saturation even at high blood PO₂s, a phenomenon known as the Root effect (e.g. Nikinmaa, 2006; de Souza and Bonilla-Rodriguez, 2007;

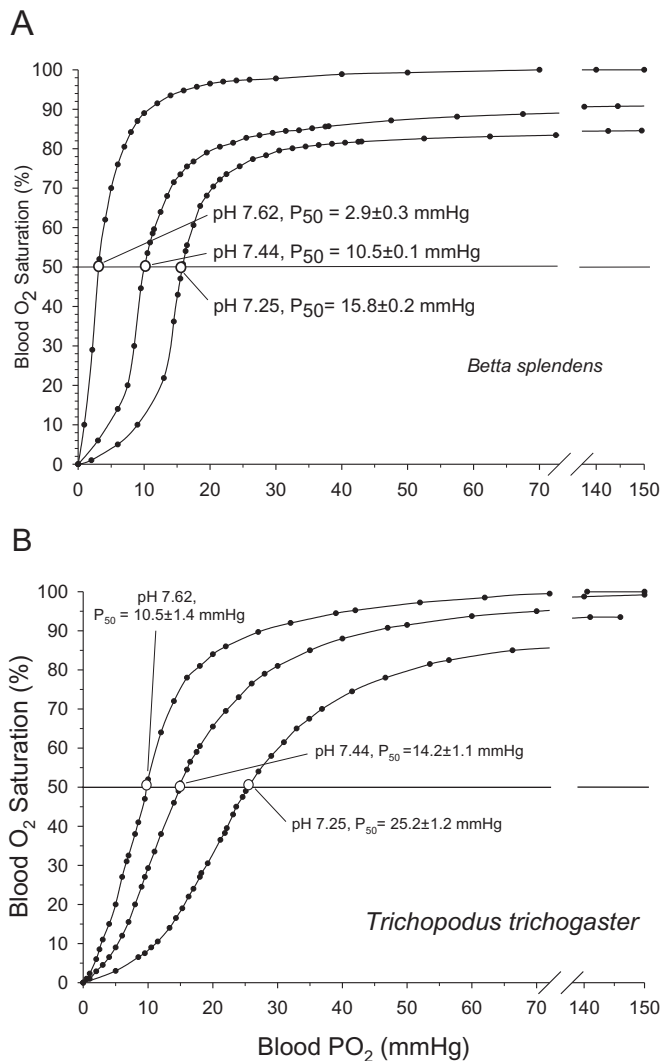


Fig. 3. Oxygen equilibrium curves of whole blood for A) the Siamese fighting fish (*Betta splendens*) and B) the blue gourami (*Trichopodus trichopterus*) at three different pH levels. Also indicated is the P_{50} for the family of curves. Each curve is a composite generated from a model based on pooled data of individual curves from six different fish for each species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Berenbrink et al., 2005; Berenbrink, 2007; Wells, 2009; Rummer and Brauner, 2015). In some marine teleosts the Root effect can produce as much as a 19% reduction in total oxygen capacity at blood air saturation and at physiological pHs (Hebert et al., 2006). Functional reasons for the evolution of large Root effects in Teleost fishes include; the ability to offload oxygen at the rete mirabile of the swim bladder to inflate this organ; creation of highly oxygenated states for the retina of the eye; and a considerable enhancement of oxygen transport to the tissues, especially when combined with a large Bohr effect (Farmer et al., 1979; Scheid et al., 1990; Pelster and Decker, 2004; Berenbrink, 2007; Randall et al., 2014; Rummer and Brauner, 2015; Hannan and Rummer, 2018).

Both anabantoid species examined in this study showed essentially full blood O_2 saturation at a pH of 7.62 and PO_2 of ~150 mmHg. A significant decrease in blood O_2 saturation at 150 mmHg developed with decreased pH in *B. splendens*. However, there was no similar Root effect in *T. trichopterus* (Table 1). In fact, lowering of blood pH to a non-physiological pH of 6.6 still failed to reveal a Root effect in the blue gourami (Herbert and Wells, 2001).

4.2. Very high blood oxygen affinity is not exceptional in air-breathing fishes

The oxygen affinity of the blood of *B. splendens* is very high compared to many other Teleosts – indeed, among the highest reported for any species. Some authors have highlighted the very high blood oxygen affinity of particular air-breathing fishes as being unusual, if not unique (e.g. Damsgaard et al., 2014; Damsgaard et al., 2015). However, a comprehensive review of ~50 years of literature on blood oxygen affinity of air-breathing fishes, including the identifies at least 16 air-breathing species having a whole blood P_{50} of < 10 mmHg, and 5 of these below 7 mmHg (Table 2). This analysis is confirmed by the review of Morris and Bridges (1994), who identified 9 of 36 air-breathing fishes with a P_{50} below 10, and 2 below 5. Thus, it would appear that high blood oxygen affinity is not exceptional in air-breathing fishes and is actually quite commonplace. Thus, the very high blood oxygen affinity of *B. splendens*, while not the rule, is certainly no exception. The blood of the carp (*Cyprinus carpio*) has a P_{50} of “just” ~ 3 mmHg (e.g. Holk and Lykkeboe, 1995; Brauner et al., 2002), and interestingly is often held up to be extraordinary for that high blood oxygen affinity. Yet, as evident from Table 1, there are at least three air-breathing fishes with similarly high oxygen affinity blood at physiological temperatures and pHs.

4.3. Advantages of a high O_2 affinity blood in air-breathing fishes

Generally, a high blood oxygen affinity is presumed to be an adaptation for fishes living in hypoxic aquatic or marine environments, allowing for efficient O_2 loading of the arterial blood as it transits the gills. Anabantoid and other air-breathing fishes have access to air, which typically raises the PO_2 of arterial blood in the ventral aorta that is destined for the gills. Air-breathing fishes with low O_2 affinity blood could face the specter of actually losing O_2 from arterial blood back out into the water flowing over the gills. A high O_2 affinity blood, coupled with the presence of non-respiratory branchial shunt vessels found in many air-breathing fishes, including the anabantoids (Burggren, 1979; Burggren and Johansen, 1986; Burggren et al., 1985; Graham, 1997), could help ensure that O_2 acquired from the air-breathing organ actually remains bound to hemoglobin as it transits the gills before it reaches the O_2 consuming systemic tissues. Relevant to this discussion of high O_2 affinity blood is the facultative nature of air breathing in *B. splendens*, compared with *T. trichopterus*, which is an obligatory air breather with a lower O_2 affinity blood. *B. splendens* can regulate to a very low the frequency of air breathing depending on its aggressive behavior and food availability (Bolds and Hogan, 1979), but this species typically breathes air if it is available. The specific circumstances under which adult *B. splendens* will suspend air breathing has not been determined in the field. Perhaps daytime hyperoxia, produced by aquatic plant photosynthesis, may suppress air breathing, as shown in the air-breathing fish *Trichopodus trichopterus* (e.g. Burggren, 1979), *Lepidosiren paradoxa* (Bassi et al., 2010), and *Protopterus dolloi* (Perry et al., 2008). These responses mirrored by the hyperoxia-induced decrease in aquatic ventilation typical of strictly aquatic fishes (e.g. Takeda, 1990; Kinkead and Perry, 1991; Porteus et al., 2015; Acharya-Patel et al., 2018).

Alternatively, the presence of or predatory fish in the water column, or predatory birds at the water surface, could result in a decrease in aerial respiration or even a switch to strictly aquatic breathing, at least temporarily (Wolf and Kramer, 1987). Another consideration is that *B. splendens* may be able to survive on aquatic respiration strictly by the gills (and skin) in an experimental situation when denied air, but does not normally do so, leading to the classification as “facultative” when in fact air breathing is rarely abandoned under natural conditions. In any event, the high blood O_2 affinity of *B. splendens*, combined with considerably larger gill surface area compared to *T. trichopterus* (Mendez-Sanchez and Burggren, in revision), is likely to enhance blood O_2

Table 2

Whole blood properties of selected air-breathing fish species with high hemoglobin oxygen affinities ($P_{50} < 10$ mmHg), arranged from lowest to highest P_{50} values.

| Species | P_{50} (mmHg) | Bohr shift | Hill's n | pH | Temp. (°C) | Reference |
|--|-----------------|--------------------|------------|------|------------|-----------------------------|
| <i>Monopterus albus</i> | 2.8 | – | 1.3 | 7.7 | 27 | Damsgaard et al. (2014) |
| <i>Betta splendens</i> | 2.9 | –2.70 ^a | 2.2 | 7.62 | 27 | Present Study |
| <i>Pterygoplichthys multiradiatus</i> | 3.5–4.7 | –0.36 | – | 7.4 | 27 | Val et al. (1990) |
| <i>Pangasianodon hypophthalmus</i> | 4.6 | – | ~2 | 7.6 | 25 | Damsgaard et al. (2015) |
| <i>Dallia pectoralis</i> ^b | 5.2 | – | – | 7.4 | 5 | Lefevre et al. (2014) |
| <i>Synbranchus marmoratus</i> | 7.0 | –0.45 | – | 7.4 | 29 | Johansen et al. (1978) |
| <i>Pterygoplichthys anisitsi</i> | 7.0 | –0.02 | 1.7 | 7.5 | 24 | Da Cruz et al. (2013) |
| <i>Synbranchus marmoratus</i> | 7.0 | –1.56 | – | 7.8 | 30 | Heisler (1982) |
| <i>Erythrinus erythrinus</i> | 7.1 | – | – | 7.4 | 29 | Johansen et al. (1978) |
| <i>Channa maculata</i> | 7.6 | –0.7 | – | 7.6 | 25 | Yu and Woo (1987) |
| <i>Monopterus cuchia</i> (Synonym <i>Amphipnous cuchia</i>) | 7.8 | –0.57 | 2.1 | 7.6 | 30 | Lomholt and Johansen (1976) |
| <i>Lepidosiren paradoxa</i> | 8.0 | –0.31 | – | 7.4 | 29 | Johansen et al. (1978) |
| <i>Amia calva</i> | 8.0 | –1.8 | – | 7.6 | 27 | Johansen et al. (1970) |
| <i>Arius leptaspis</i> | 9.0 | –0.22 | 2.15 | 7.4 | 25 | Wells et al. (2005) |
| <i>Electrophorus electricus</i> | 9.7 | –0.43 | 1.6–2.45 | 7.4 | 29 | Johansen et al. (1978) |

^a Mean of values over three pH ranges from 7.62–7.25.

^b blood hemolysate rather than whole blood.

transport in any condition when air breathing is not available. While the transition to mixed air breathing and water breathing can be modified by environmental hypoxia or hyperoxia in *B. splendens* and *T. trichopterus* (Mendez-Sanchez and Burggren, 2017), the specific environmental conditions that might cause such switching between strictly aquatic and mixed aquatic and aerial in *B. splendens* have not been determined, and would be an interesting topic for future study.

A final consideration for the explanation of the potential differences between *B. splendens* and *T. trichopterus* are the differences in lifestyle. A gradient of fish lifestyles has been proposed by Wells et al. (2005), ranging from lethargic, hypoxic-tolerant fishes to those fishes that are highly active and primarily inhabit highly oxygenated water. Lethargic, hypoxia-tolerant fishes often present smaller P_{50} values (higher blood O_2 affinity), show larger Bohr and Root effects and larger increases in O_2 -hemoglobin cooperativity (higher values of Hill's n). All of these characteristics are evident in *B. splendens*. On the other hand, *Trichopodus trichopterus* showed higher P_{50} and other blood O_2 binding characteristics, anticipated for an active fish less adapted to environmental functional hypoxia. Indeed, at least for their larvae and juveniles, *B. splendens* shows considerable developmental physiological and developmental plasticity in aid of acquiring O_2 as a result of chronic hypoxia, compared with the more muted responses (at least of the gills) for *T. trichogaster* (Blank and Burggren, 2014; Mendez-Sanchez and Burggren, 2017; Mendez-Sanchez and Burggren, in revision).

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Declarations of interest

None.

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