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RESEARCH ARTICLE

Hypoxia-induced developmental plasticity of larval growth, gill and labyrinth organ morphometrics in two anabantoid fish: The facultative air-breather Siamese fighting fish (*Betta splendens*) and the obligate air-breather the blue gourami (*Trichopodus trichopterus*)

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Abstract

Larval and juvenile air breathing fish may experience nocturnal and/or seasonal aquatic hypoxia. Yet, whether hypoxia induces respiratory developmental plasticity in larval air breathing fish is uncertain. This study predicted that larvae of two closely related anabantid fish—the facultative air breather the Siamese fighting fish (*Betta splendens*) and the obligate air breathing blue gourami (*Trichopodus trichopterus*)—show distinct differences in developmental changes in body, gill, and labyrinth morphology because of their differences in levels of dependency upon air breathing and habitat. Larval populations of both species were reared in normoxia or chronic nocturnal hypoxia from hatching through 35–38 days postfertilization. Gill and labyrinth variables were measured at the onset of air breathing. *Betta splendens* reared in normoxia possessed larger, more developed gills (~3× greater area) than *T. trichopterus* at comparable stages. Surface area of the emerging labyrinth, the air breathing organ, was ~ 85% larger in normoxic *B. splendens* compared to *T. trichopterus*. Rearing in mild hypoxia stimulated body growth in *B. splendens*, but neither mild nor severe hypoxia affected growth in *T. trichopterus*. Condition factor, K (~ 1.3 in *B. splendens*, 0.7 in *T. trichopterus*) was unaffected by mild hypoxia in either species, but was reduced by severe hypoxia to <0.9 only in *B. splendens*. Severe, but not mild, hypoxia decreased branchial surface area in *B. splendens* by ~40%, but neither hypoxia level affected *Trichopodus* branchial surface. Mild, but not severe, hypoxia increased labyrinth surface area by 30% in *B. splendens*. However, as for branchial surface area, labyrinth surface area was not affected in *Trichopodus*. These differential larval responses to hypoxic rearing suggest that different larval habitats and activity levels are greater factors influencing developmental plasticity than genetic closeness of the two species.

KEYWORDS
development, hypoxia, morphological plasticity, respiratory surface area

1 | INTRODUCTION

Hypoxia is a common environmental stressor in aquatic animals. In Teleost fish hypoxia may have multiple effects, including alteration of food intake, rate of development and growth, stimulation of ventilatory and cardiovascular reflexes, and ultimately mortality as a consequence (Johansen & Lenfant, 1968; Burggren, 1982; Burggren & Johansen, 1986; Perry, 2011; Milsom, 2012; Porteus, Hedrick, Hicks, Wang, & Milsom, 2011; Martin, 2014; Blank & Burggren, 2014; Mendez-Sanchez & Burggren, 2014; Abdallah, Thomas, & Jonz, 2015; Burggren et al., 2014; Mendez-Sanchez & Burggren, 2017). Responses to hypoxia appear to be specific to each species, even when comparing relatively closely related species (Farrell & Richards, 2009; Mendez-Sanchez & Burggren, 2014),

Growth limitations induced by hypoxia in fish can occur through a reduction in appetite. This response and the associated growth retardation occurs at relatively mild hypoxia levels (Wang, Lefevre, Huong, Cong, & Bayley, 2009). As examples, juveniles of the turbot *Scophthalmus maximus* and the sea bass *Dicentrarchus labrax* were fed to satiation under hypoxia, and their growth and food intake were depressed compared to those raised in normoxia (Pichavant et al., 2001). In the juvenile catfish *Silurus meridionalis*, growth performance was also impaired due to acclimation to dial cycling hypoxia (Yang, Cao, & Fu, 2013). Juvenile piapara (*Leporinus elongates*) exposed to severe hypoxia also showed lower weight gain. Moreover, feed conversion, that is, the quantity of food consumed divided by the weight gain over a given a time period, was 1.6 when compared to fish in moderate hypoxia (2.9) and in normoxia (1.8); (Filho, Torres, Zaniboni-Filho, & Pedrosa, 2005). Variable patterns of the relationship between growth and hypoxia occur among air-breathing fish. For example, juveniles of the facultative air breather *Pangasionodon hypophthalmus* experience minor losses in growth performance (Phuong, Huong, Nyengaard, & Bayley, 2017) in contrast to juveniles of the more air-reliant *Chitala ornata* that continues with unimpaired growth even when reared in hypoxia (Tuong et al., 2018).

Chronic hypoxic exposure not only retards overall growth, but also affects size and structure of fish respiratory organs in strictly aquatic species of fish. Hypoxic modulation of branchial structures has been observed in strictly water breathing fish such as the Sailfin molly *Poecilia latipinna*, four species of knife fish *Brachyhypopomus*, the Nile perch *Lates niloticus*, the Crucian carp *Carassius carassius*, and the goldfish *Carassius auratus*. All of these increased gill lamellar surface area and filament length in response to chronic hypoxia (Crampton, Chapman, & Bel, 2008; Nilsson, 2007; Paterson, Chapman, & Schofield, 2010; Timmerman & Chapman, 2004).

The effects of hypoxia on growth rate and branchial structure have been studied in a few species of air-breathing fish (e.g., Lefevre et al., 2014; Phuong et al., 2017). It might be anticipated that obligate air-breathing fish are unlikely to be strongly affected by aquatic hypoxia, given a presumed source of aerial oxygen. Indeed, in the obligate air breathing gourami *Trichopodus trichopterus*, mild hypoxia had no effect on wet body mass, although lamellar and labyrinth surface areas increased more rapidly in larvae developing in hypoxia (Blank & Burggren, 2014). In contrast, juveniles of the obligate air breather *Pangasianodon hypophthalmus* showed considerable gill hypertrophy with chronic hypoxic rearing (Phuong et al., 2017). Facultative air-breathing fish, on the other hand, appear to be negatively affected by aquatic hypoxia and the few existing studies point to clear negative effects on body growth and gill development and surface area in these facultative air-breathing fish (Turko, Cooper, & Wright, 2012; Wang et al., 2009). For example, in the obligate air-breathing striped snakehead *Channa striata* (synonym of *Ophicephalus striatus*), the cost of compensating for low aquatic oxygen availability by increasing surfacing and air-breathing frequency actually exceeds the gain in food conversion that occurs at higher tissue oxygen levels (Kramer & McClure, 1981). This situation is further complicated by the fact that hypoxia reduced food consumption (Pandian & Vivekanandan, 1976; Vivekanandan, 1977; Wang et al., 2009). Similarly, in the facultative air-breathing stinging catfish, *Heteropneustes fossilis*, the cost of surfacing for air breathing exceeds the gain in body mass it might otherwise afford. With further hypoxia, it is more efficient for this species to rely solely on aquatic respiration rather than expend additional energy in air breathing (Arunachalam, Vivekanandan, & Pandian, 1976; Wang et al., 2009).

Juvenile air-breathing fish (facultative or obligate) tend to depend on aquatic respiration (both cutaneous and branchial) until the air-breathing organ has sufficiently developed (Brauner, Matey, Wilson, Bernier, & Val, 2004; Burggren et al., 2016; Wang et al., 2009). While the effects of hypoxia on larvae of air-breathing fish are largely unknown, a few studies have shown hypoxia to slow the transition to air breathing as well as body growth itself. For example, in the paradisefish *Macropodus opercularis*, the air-breathing organ develops more slowly under hypoxic conditions (Ebeling & Alpert, 1966). The onset of air breathing is greatly delayed in juveniles of the obligate anabantid air-breath three spot gourami *Trichopodus trichopterus*, by exposure to 13% hypoxia (Mendez-Sanchez & Burggren, 2014). This level of hypoxia also alters the allometric relationships between the wet weight and the branchial arches and the total labyrinth surface area in this species (Blank & Burggren, 2014).

Hypoxia induced high mortality in the pre air-breathing larva of *Betta splendens* and *Trichopodus trichopterus* (Mendez-Sanchez & Burggren, 2014). Continuous hypoxia (14–17 kPa of PO_2) was lethal to the young larvae of both species beyond 9 days postfertilization (dpf). However, nocturnal intermittent exposure to hypoxia resulted in 75% mortality in both *Betta splendens* larvae and 80% in *T. trichopterus* beyond 35 and 38 dpf, respectively. These results highlight dissolved oxygen as a limiting environmental factor for larval fish in fresh water (Diaz & Breitburg, 2009). It also reinforces hypoxia as a strong natural selection force as in adult air-breathing fish (Graham, 1997; Randall, Burggren, Haswell, & Farrell, 1981), with a high cost evident in lowered larval survival.

The current study expands this meagre knowledge of developmental plasticity in the face of hypoxia in air-breathing fish. We chose the three spot gourami *Trichopodus trichopterus* as an obligate air-breather (Blank, 2009; Burggren, 1979; Burggren & Blank, 2009; Graham, 1997; Herbert & Wells, 2001). The genus *Trichopodus* is a target for aquatic aquaculture for both food and the ornamental market (Jafaryan, Sahandi, & Dorbadam, 2014), making the understanding of how hypoxia affects larval growth in this genus particularly useful, just as such understanding in other species subject to aquaculture may contribute to that industry's success (e.g., Phuong et al., 2017). *Betta splendens* was chosen as an example of a facultative air breather (Graham, 1997; Peters, 1978). Both species are anabantoid labyrinth fish from the Osphronemidae, with *Trichopodus trichopterus* from the subfamily Luciocephalinae and *Betta splendens* from the subfamily Macropodusinae (Froese & Pauly, 2018). However, the Siamese fighting fish lives a more sedentary lifestyle carried out in more hypoxic waters than the gourami (Rainboth, 1996; Rüber, Britz, & Zardoya, 2006; Monvises, Nuangsaeng, Sriwattanarothai, & Panijpan, 2009; Froese & Pauly, 2018).

Against this backdrop, the goals of this study of larval *Betta splendens* and *Trichopodus trichopterus* were to: (a) determine the basic changes in gill morphometrics from early development to the air breathing stage; (b) assess the effect of intermittent nocturnal hypoxia on growth (size and weight) in larvae and; (c) quantify how chronic hypoxic exposure alters lamellar and labyrinth respiratory surfaces. We expected that developmental plasticity of the gills and labyrinth would relate more closely to the habitat and dependence on air breathing than it would to the genetic similarities of these two closely related species. Additionally, we expected that the larvae of *B. splendens*, the facultative air-breather, would exhibit greater respiratory developmental plasticity than the larvae of *T. trichopterus*, the obligate air-breather, thus allowing *B. splendens* to be more tolerant of a hypoxic environment.

2 | MATERIALS AND METHODS

2.1 | Animals

Experiments were conducted on selected stages of pre-air-breathing larval *Betta splendens* Regan, 1910 and *Trichopodus trichopterus* (Pallas 1770) (formerly *Trichogaster trichopterus*) ranging from 0 days to the onset of breathing—38 dpf in *B. splendens*, and 35 days in *T. trichopterus*. The breeding and handling of larva and adults of both species was approved by the University of North Texas Animal Care and Use Committee with protocol number 1111-16.

2.2 | Rearing protocols

Details of fish breeding, rearing protocol, and water chemistry have been provided in previous reports (Mendez-Sanchez & Burggren, 2014, 2017). Briefly, approximately 150 larval *B. splendens* and 300 larval *T. trichopterus*, each from a single clutch to minimize genetic variation, were maintained at 27°C from hatching to 35 dpf for *T. trichopterus* or 38 dpf for *B. splendens*. Day of fertilization was designated 0 dpf. For the first 48 hr following hatching all larvae were reared in normoxia. Larvae of both species were then moved to floating containers (250 mL) in 40 L aquaria. Larvae of each species were raised in either continuous normoxia at a PO_2 of 20 kPa or in intermittent nocturnal hypoxia (17 and 14 kPa of PO_2) until 35 dpf (*T. trichopterus*) or 38 dpf (*B. splendens*). Both 17 and 14 kPa levels of hypoxia result in significant physiological and morphological changes in larvae of these species (Blank, 2009; Blank & Burggren, 2014; Mendez-Sanchez & Burggren, 2014, 2017). Intermittent hypoxia exposure, consisting of 12 hr of the desired hypoxia level during the dark phase of the 24 cycle, and 12 hr of daytime normoxia, was used for two reasons. First, this protocol mimicked naturally occurring nocturnal hypoxia. Moreover, previous studies showed that even mild hypoxia was lethal when continuously applied in the early larvae of these two species (Mendez-Sanchez & Burggren, 2014).

A flow of either room air (control, PO_2 ~ 20 kPa) or a mixture of room air and nitrogen gas was passed through air stones in the bottom of each water container, creating hypoxia at levels of either 17 kPa or 14 kPa. Gas flows were regulated with flowmeters set to deliver the appropriate gas mixture. The aquaria with the floating containers were sealed with Plexiglass covers to prevent atmospheric air from leaking into the containers. Eighty percent of the container contained water, with the top 20% of the container consisting of the gas emerging up from air stone in the water below. Larvae had free access to the surface, although most of the measurements were completed in the strictly aquatic phase of gas exchange prior to the onset of air breathing around day 35–38 of development. Gas and water phases of the containers were in PO_2 equilibrium, ensuring that the larvae were exposed only to a specific gas level. An optical oximeter probe ProODO (YSI Incorporated) and a ProOx 110 oxygen sensor (Biospherix, Ltd, Parish, NY, USA) were used to monitor water and air PO_2, respectively, on a daily basis.

2.3 | Measurements

2.3.1 | Body length, mass, and condition factor

Every five dpf five larvae of each treatment were euthanized by moving them into buffered MS222 solution (pH = 7.0) until well after opercular movement stopped, indicating death. Sampling of *B. splendens* only started on 20 dpf because of the small clutch size (~150 eggs) and to ensure enough animals at the end of 35 dpf period for the gill and labyrinth morphometrics analysis. Body length (snout to end of tail) was measured to the nearest 10 μm using a microscope. Wet mass was collected for each larva to the nearest 0.1 mg. To do so, each larva was first placed on a Kimwipe® first on its left side, then its right, to carefully absorb excess water from the body surface and buccal and opercular cavities. The larva was then transferred to a dry, tared metal scale, and the surface of the scale again blotted to absorb any remaining water.

Fulton's condition factor (K) was calculated from the formula:

$$K = BM/L^3$$

where BM = fish body mass (g), and L = fish body length (mm). Noteworthy is that more detailed measures of body condition assuming non-isometric growth have been proposed – (e.g., Richter, Lückstädt, Focken, & Becker, 2000). However, the large difference in K between *B. splendens* and *T. trichopterus* in the current study suggested that Fulton's K was sufficient for comparative purposes when employed over the relatively small range of growth during early development.

2.3.2 | Gill and labyrinth measurements

Measurements were made on larvae at 38 dpf (*B. splendens*) or 35 dpf (*T. trichopterus*), which represents the approximate time of onset of air breathing). In younger larva it is difficult to visualize either lamellae on the filaments or the labyrinth organ on the fourth branchial arch. After measurement of body length and mass, larvae were fixed in a 10% neutral buffered formalin solution. After 10 days of fixation, the gills, and the labyrinth organ of five individuals per treatment of each species were dissected out, and photographed with a 10-megapixel-camera Leica model DFC450 using a compound microscope Leica model Wild M3Z. From the images, counts made of lamellae and branchial filaments and lengths and areas measured by using Image J 1.42 software (http://rsbweb.nih.gov). All areas and lengths were calibrated in pixels with the scale of a micrometer slide.

Total lamellar respiratory surface (TSA_{lam}) and total labyrinth organ respiratory surface (TSA_{lab}) were calculated for 35 dpf larvae

(*T. trichopterus*) and 38 dpf larvae (*B. splendens*) . TSA_{lam} was obtained after the methods of Hughes (1984) and Blank and Burggren (2014). Average lamellar area ($\bar{X}SA_{lam}$) was determined using the mean of the lamella area in the medial position on one proximal, one medial, and one distal gill filament for each of the four branchial arches. To account for both lamella surfaces, each calculated lamellar area was doubled. The number of total lamella in each arch was multiplied by $\bar{X}SA_{lam}$ to obtain lamellar surface area per arch, designated as SA_{lamI}, SA_{lamII}, SA_{lamIII}, and SA_{lamIV}. Finally, total lamellar surface area ($TSA_{lamella}$) was determined as the sum of SA_{lamI}, SA_{lamII}, SA_{lamIII}, and SA_{lamIV}, multiplied by two to account for both sides of the gills.

The primordial labyrinth at the onset of air breathing in *T. trichopterus and B. splendens* is not the complex multifaceted structure of the adult. Rather, it first appears as a small, simply structured ridge-like elevation on dorsal side of the fourth branchial arch (Blank, 2009; Blank & Burggren, 2014). While likely not representing a highly functional respiratory surface area at the onset of air breathing, we nonetheless measured the total surface area of the emerging labyrinth (TSA_{lab}) to determine whether the size of the emerging labyrinth differed after larvae have been raised in normoxia compared with hypoxia. TSA_{lab} was calculated by first tracing the outline of the elevated profile of the ridge-like labyrinth organ when viewed from the side. We then quantified the profile's surface area in a scaled photo. The measured surface area of the emerging labyrinth ridge was then doubled to account for the two sides of the labyrinth respiratory surface, and also doubled again to account for left and right labyrinth organs.

Gill arch length (L_{arch}) of the four branchial arches (I, II, III, and IV), as well as filament length (L_{flm}) on each arch, were compared between species and PO_2 levels. Also compared were the number of filaments per arch (NF_{arch}), number of lamellae per filament (NL_{flm}), and single lamella surface area (SA_{lam}). For each branchial arch, these variables were measured on three filaments: anterior filament (A), medial filament (M), and posterior filament (P), except on the IV arch for which only A and P filaments were measured because of the small size and low number of filaments for this arch. Branchial arch nomenclature was based on extant branchial arches in mature fish, namely Arch I, II, III and IV.

Finally, mass-specific TSA_{lam} and mass-specific TSA_{lab}, expressed in mm^2/mg body mass, were calculated by dividing TSA_{lam} and TSA_{lab} by body mass, all measured at the onset of air breathing.

2.4 | Statistical analysis

ANOVA and MANCOVA comparisons were performed on wet mass, body length, TSA_{lam}, TSA_{lab}, L_{arch}, NF_{arch}, L_{flm}, NL_{flm}, and SA_{lam} using species and PO_2 level as factors. On the MANCOVA comparisons, body length was treated as a covariate to minimize the effect of different body sizes. The treatment groups were considered significantly different when the *p*-value was lower than .05.

3 | RESULTS

3.1 | Basic respiratory variables and their development in normoxia

3.1.1 | Body length, wet mass, and condition factor in normoxia

Average body length for larval *Betta splendens* in normoxia increased from 5.6 mm at 20 dpf to 8.9 mm at 35 dpf (Figure 1). Both age and PO_2 treatment were significant effects on body length in *B. splendens* (F_{age} = 13.8, *df* = 4, 64 and $p < .0001$; F_{PO2} = 3.83, *df* = 4, 64 and $p < .05$). For *T. trichopterus* reared in normoxia, average body length increased from 3.2 mm at 5 dpf to 7.8 mm at 35 dpf (Figure 2) (F_{age} = 51.92, *df* = 7, 83, and $p < .0001$).

Average body wet mass for larval *B. splendens* in normoxia was 2.26 mg at 20 dpf, increasing to 5.5 mg at 35 dpf (Figure 3). Both age and PO_2 treatment (see below) significantly altered body wet mass (F_{age} = 6.63, *df* = 4, 64, $p < .001$). *T. trichopterus*, as expected, also

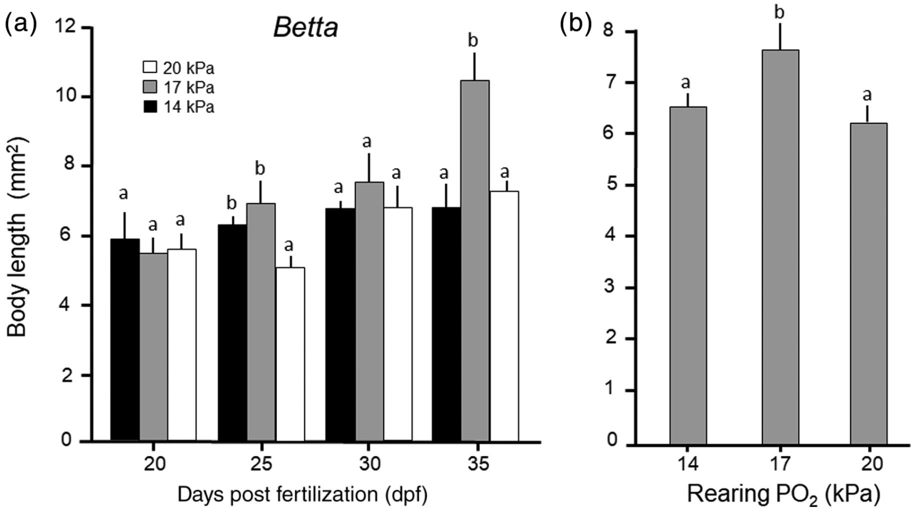

FIGURE 1 Body length as a function of rearing PO_2 in larval *Betta splendens*. (a) Body length through development in each PO_2 treatment. (b) Effect of PO_2 on body length. Body length was treated as a covariate in a MANCOVA to minimize the effect of body mass differences associated with different developmental stages (see statistical methodology described in text). Means and positive standard errors are presented. Letters indicate intraspecific statistics of differences of means ($p < .05$). $n = 53$

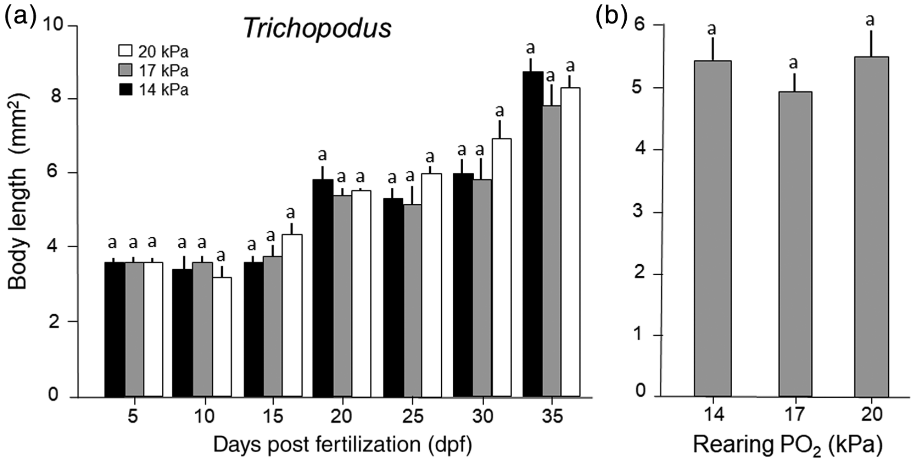

FIGURE 2 Body length as a function of rearing PO_2 in larval *Trichopodus trichopterus* (a) Body length through development in each PO_2 treatment. (b) Body length corrected for the effect of age (see statistical methodology described in text). Means and positive standard error are presented. Letters indicate intraspecific statistics of differences of means ($p < .05$). $n = 85$

increased their body wet mass through development ($F_{age} = 29.58$, $df = 7, 83$, $p < .0001$) (Figure 4).

The condition factor for *B. splendens* was ~1.3 when reared in normoxia, compared with only ~0.7 in normoxic *T. trichopterus* (Figure 5).

3.1.2 | Gill arch morphometrics in normoxia

Gill arch length decreased significantly with ascending arch number in both species ($F = 100$, $df = 1, 71$ and $p < .05$; Figure 6), falling from about 0.9 mm and 1.3 mm for Arch I in *B. splendens* and *T. trichopterus*, respectively, to 0.5 mm and 0.95 mm on gill Arch IV. *B. splendens* had significantly longer gill arches than *T. trichopterus* ($F = 7.31$, $df = 1, 71$ and $p < .05$).

Betta splendens had significantly more filaments per arch than *T. trichopterus* ($F = 18.87$, $df = 1, 71$ and $p < .05$), particularly in the second, third, and fourth arches which are anatomically distinct from the labyrinth organ (Figure 7).

In both species, there were distinct differences in filament length as a function of gill arch number (Figure 8). Only short filaments were evident on the fourth branchial arch, and filaments were actually still absent in the medial region in both species. For extant gill filaments, filament lengths were significantly longer in *B. splendens* than in *T. trichopterus* on all the branchial arches ($F = 7.3$, $df = 2, 71$ and $p < .05$), when L_{flm} was corrected for body length.

Betta splendens had 20–40% more lamella per filament than *T. trichopterus* ($F = 454$, $df = 1, 215$ and $p < .05$) on all four gill arches (Figure 9).

Single lamella area per filament per arch (SA_{lam}) in *B. splendens* was nearly double that of *T. trichopterus* in normoxia ($F = 107$, $df = 2, 197$ and $p < .05$), when SA_{lam} was corrected for body length (Figure 10).

3.1.3 | Respiratory surface areas in normoxia

Total surface area of all lamellae (TSA_{lam}) of *B. splendens* was on average more than 3–4 times larger than in *T. trichopterus* when corrected for body length ($F = 69.8$, $df = 1, 17$ and $p < .05$; Figure 11a). Data expressed on a mass-specific basis are provided in Table 1.

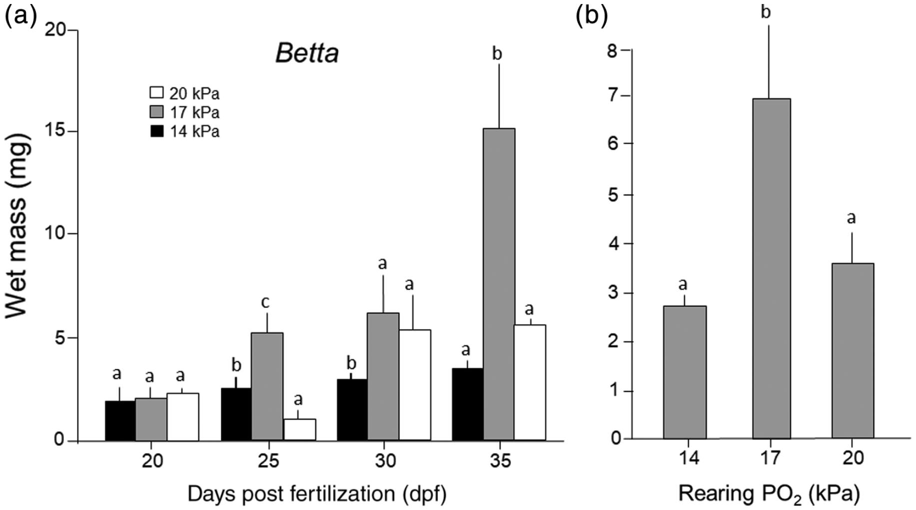

FIGURE 3 Wet body mass as a function of rearing PO_2 in larval *Betta splendens*. (a) Wet mass through development in each PO_2 treatment. (b) Wet mass corrected for the effect of age (see statistical methodology described in text). Means and positive standard error are presented. Letters indicate intraspecific statistics of differences of means ($p < .05$). $n = 53$

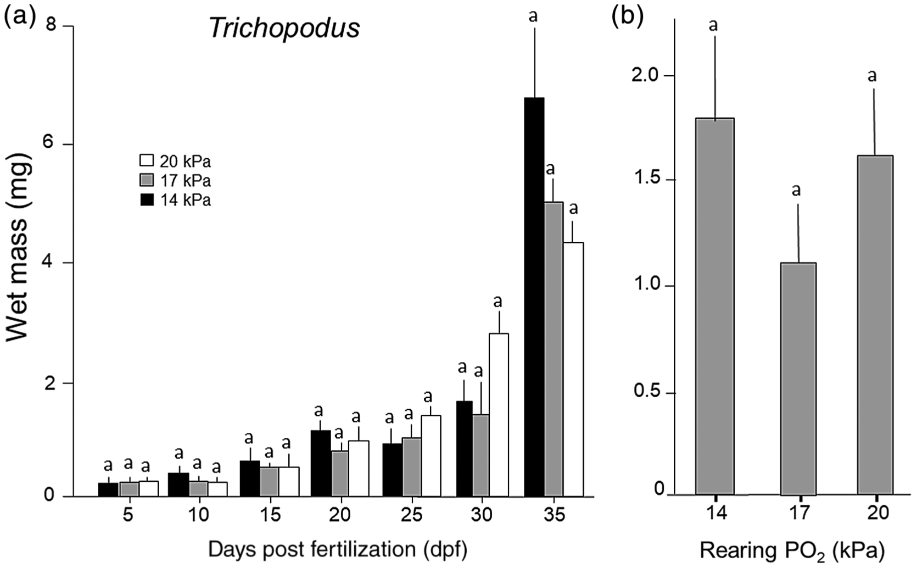

FIGURE 4 Wet body mass as a function of rearing PO_2 in *Trichopodus trichopterus*. (a) Larval wet mass through development in each PO_2 treatment. (b) Larval wet mass correcting for the effect of age (see statistical methodology described in text). Means and positive standard error are presented. Letters indicate intraspecific statistics of differences of means ($p < .05$). $n = 85$

Total labyrinth surface area (TSA_{lab}) for *B. splendens* at ~38 dpf reared in normoxia was 0.25 mm^2 (Figure 11b, Table 1). TSA_{lab} for *T. trichopterus* reared in normoxia was significantly lower at just $0.07 mm^2$, ($F = 69.79$, $df = 2, 17$, $p < .05$).

3.2 | Influence of hypoxia

3.2.1 | Body length, wet mass, and condition factor in hypoxia

Betta splendens exposed to a PO_2 of 17 kPa were 1.45 times longer than the normoxic group. In contrast, in *T. trichopterus*, rearing $P0_2$ did not significantly affect body length ($F_{PO2} = 1.96$, $df = 2, 83$, and $p > .05$).

PO_2 treatment significantly altered body wet mass ($F_{PO2} = 3.53$, $df = 2, 64$, $p < .05$) in *B. splendens*. Specifically, at the onset of air-breathing wet body mass corrected for age was 50% greater in hypoxia at 17 kPa compared to the normoxic *B. splendens* group (Figure 3b). For *T. trichopterus* rearing PO_2 had no effect on body mass ($F_{PO2} = 1.45$, $\underline{df} = 2, 83$, $p > .05$; Figure 4). Collectively, these results

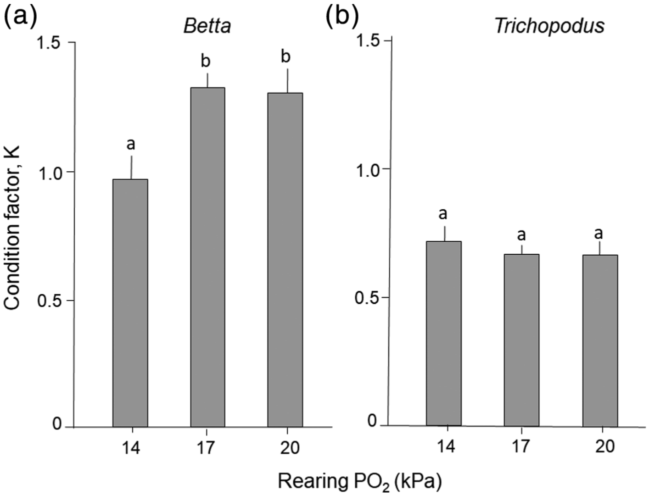

FIGURE 5 Condition factor (K), corrected for age, as a function of rearing PO_2. (a) *Betta splendens* (38 dpf). (b) *Trichopodus trichopterus* (35 dpf). Means and positive standard error are presented. Letters indicate intraspecific statistics of differences of means ($p < .05$)

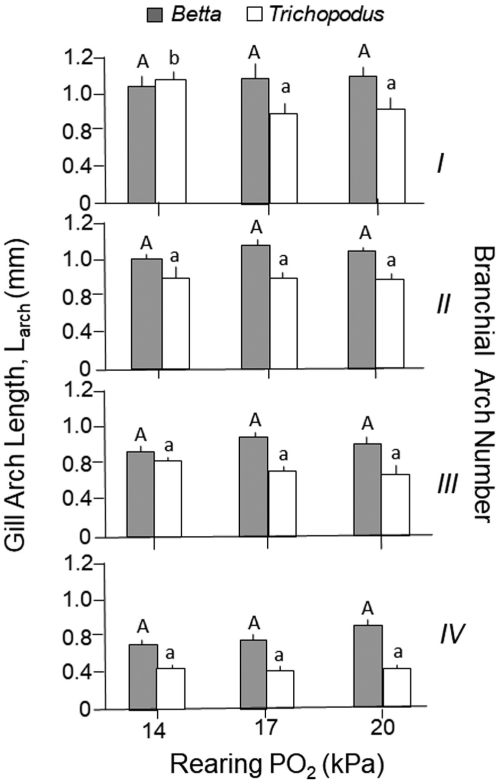

FIGURE 6 Gill arch length (L_{arch}) respiratory surface areas at the onset of air-breathing as a function of rearing PO_2 in *Betta splendens* (38 dpf) and *Trichopodus trichopterus* (35 dpf). Means and positive standard errors are presented. Letters (capital for *B. splendens*, small for *T. trichopterus*) indicate intraspecific statistics of differences of means, corrected for body length. $n = 9$ for *B. splendens*, $n = 9$ for *T. trichopterus*

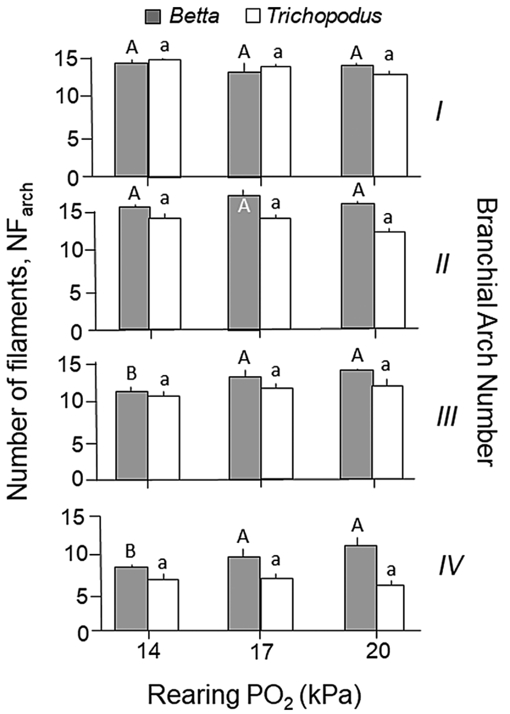

FIGURE 7 Number of filaments per arch (NF_{arch}) at the onset of air-breathing at the onset of air-breathing as a function of rearing PO_2 in *Betta splendens* (38 dpf) and *Trichopodus trichopterus* (35 dpf). Means and positive standard errors are presented. Letters (capital for *Betta*, small for *T. trichopterus*) indicate intraspecific statistics of differences of means, corrected for body length. $n = 9$ for *B. splendens*, $n = 9$ for *T. trichopterus*

showed that in *B. splendens* only hypoxia at 17 kPa of PO_2 increased body wet mass, and in *T. trichopterus* neither hypoxic level significantly affected body wet mass during development.

The condition factor for *B. splendens* in mild hypoxia group was ~1.3 but was significantly lower (<1.0) in the most hypoxic group at 14 kPa ($F = 4.93$, *df* = 2, 64, $p < .05$; Figure 5a). For *T. trichopterus*, the condition factor, ~0.7, was not significantly affected by rearing oxygen level ($F = 2.9$, *df* = 2, 107, and $p > .05$;Figure 5b).

3.2.2 | Gill morphometrics in hypoxia

Hypoxic rearing at either level did not significantly affect gill filament length in either species, when analyzed with MANCOVA using body length as a covariate, ($F = 0.41$, *df* = 2, 71, and $p > .05$; Figure 8). Similarly, hypoxic rearing did not induce any significant differences within either species ($F = 0.13$, *df* = 2, 71, $p > .05$). *Trichopdus trichopterus* also showed no changes in NF_{arch} as a function of rearing PO_2 (Figure 7). However, in *B. splendens* hypoxic rearing produced a significant decrease in number of filaments of the third and fourth arches for the 14 kPa treatment ($F = 9.9$, *df* = 2, 71, and $p < .05$).

Hypoxic rearing at either level produced a slight but significant increase in number of lamellae per filament per arch on the medial and anterior sections of the first filament in *B. splendens* ($F = 3.3$, *df* = 2, 215, and $p < .05$; Figure 9). In contrast, *T. trichopterus* showed no significant changes in NL_{flm} related with the PO_2 treatment.

SA_{lam} decreased significantly in *B. splendens* when reared at 14 kPa ($F = 2.8$, *df* = 2, 197, and $p < .05$). However, larval *T. trichopterus* showed no significant changes in SA_{lam} related to PO_2 treatment (Figure 10).

3.2.3 | Respiratory surface areas in hypoxia

Total branchial lamellar surface area in *B. splendens* was unchanged by mild hypoxia, but when reared in more severe hypoxia of 14 kPa (Figure 11a), TSA_{lam} showed a significant, approximately 40% decrease in TSA_{lam} from ~25 mm^2 to ~15 mm^2 ($F = 10.48$, df = 2, 8, and $p < 0. 05$). However, in *T. trichopterus* hypoxic exposure did not significantly affect lamellar respiratory surface ($F = 0.52$, df = 2, 8 and $p > .05$). Mass-specific values for total branchial lamellar surface are presented in Table 1.

Rearing at a $P0_2$ 17 kPa increased labyrinth respiratory surface in *B. splendens* by 30% compared with the normoxic group ($F = 11.14$, *df* = 2, 8, and $p < .05$), when corrected for the effect of body length (Figure 11b). Rearing PO_2 level in *T. trichopterus*, unlike in *B. splendens*, did not significantly alter TSA_{lab} ($F = 0.33$, *df* = 2, 8, and $p > .05$;) (Figure 11b). Mass-specific values for total labyrinth surface areas are presented in Table 1.

4 | DISCUSSION

4.1 | Larval growth patterns

The predicted differences between larvae of *B. splendens* and *T. trichopterus* in their responses to hypoxic exposure were evident in their distinctive growth patterns. Larval *B. splendens* showed a significant growth increase in both body size and wet mass when they were exposed to mild hypoxia (17 kPa), but this was reversed by severe hypoxia (14 kPa of PO_2), which diminished the condition factor of *B. splendens*. In contrast, in larval *T. trichopterus* no growth variable was significantly altered by hypoxia, as similarly reported for this species (Blank, 2009; Blank & Burggren, 2014). Interestingly, there was larger variation in length and mass within a single age and PO_2 cohort in *B. splendens* compared to *T. trichopterus*. That is, *B. splendens* showed a greater developmental plasticity than *T. trichopterus*. Collectively, these data reveal the higher sensitivity of *B. splendens* to aquatic hypoxia and the absence of response of growth variables of *T. trichopterus* to the same environmental stressor.

Decreased growth of air breathing fish under hypoxic conditions observed by Wang et al. (2009) and Filho et al. (2005) also occurred in larval *B. splendens* reared in severe hypoxia, as evident from this species' lower condition factor. However, when the data were additionally analyzed to consider the increase of weight and size as a factor of age, mild hypoxia enhanced both growth variables. In contrast, larval *T. trichopterus* did not follow this pattern with all conditions including normoxia, being delayed relative to that in *B. splendens*. *Trichopodus trichopterus* appears unable to mount an adaptive morphological response at this point of development, resulting in high mortality in both experimental groups (Mendez-Sanchez & Burggren, 2014). This higher mortality could be compensated for by the considerably higher number of eggs (2,000–3,000) in a single clutch compared to the

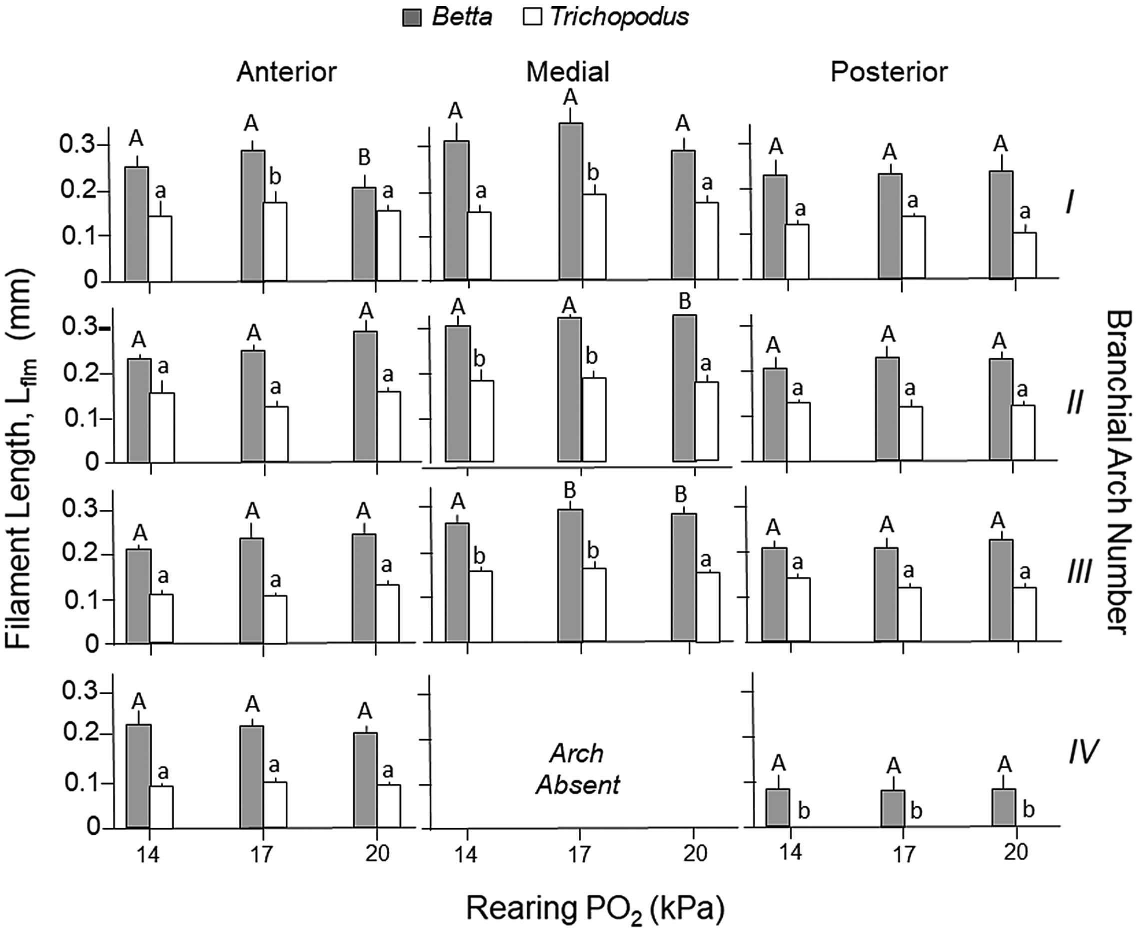

FIGURE 8 Filament length (L_{flm}) at the onset of air-breathing as a function of rearing PO_2 in *Betta splendens* (38 dpf) and *Trichopodus trichopterus* (35 dpf). Means and positive standard errors are presented. Letters (capital for *Betta*, small for *T. trichopterus*) indicate intraspecific statistics of differences of means, corrected for body length. $n = 9$ for *B. splendens*, $n = 9$ for *T. trichopterus*

300–500 eggs laid in a clutch by *B. splendens* (Clotfelter, Curren, & Murphy, 2006; Froese & Pauly, 2018; Monvises et al., 2009).

4.2 | Gills, labyrinth organ, and their developmental plasticity

4.2.1 | Normoxic branchial and labyrinth development

Betta splendens at the onset of air-breathing (38dpf) in normoxia had larger gills than *T. trichopterus*, as evident in gill arch length, number of filaments and lamellar density on each filament. As a consequence, the total lamellar surface area of *B. splendens* at the onset of air-breathing (38 dpf) was nearly five times larger than *T. trichopterus* at 35 dpf (Figure 11a). Similarly, *B. splendens* exhibited a total labyrinth surface area nearly four times larger (Figure 11b). At the onset of air breathing, *B. splendens* is heavier and longer, but the larger size of *B. splendens* is insufficient to account for these respiratory surface area differences.

These findings confirm our original predictions that, despite both being anabantoids, the quantitative design of their branchial morphology would differ as a consequence of the different environments they inhabit and the natural histories they exhibit. The context for these differences is discussed below, after consideration of the labyrinth organ and its developmental plasticity in the two species.

4.2.2 | Branchial responses to chronic hypoxia

Larval *B. splendens* showed considerable morphological developmental plasticity. Interestingly, there was a relationship between rearing PO_2 and total lamellar surface area (TSA_{lam})—that is, severe hypoxia inhibited the development of the branchial respiratory surfaces through reduced total lamellar surface area. This is in contrast to the branchial hypoxic response occurring in the cyprinids and some other fish, where there is an adaptive response in which TSA_{lam} increases as hypoxia deepens (Crampton et al., 2008; Nilsson, 2007; Paterson et al., 2010; Timmerman & Chapman, 2004). Some of these changes are known to occur on a time scale of a few days (Sollid, De Angelis, Gundersen, & Nilsson, 2003). All fish in these studies were adult water-breathers exposed to acute hypoxia treatment. This differs from the current study, where the larvae were actually reared in chronic intermittent hypoxia. In numerous adult air breathing fish, branchial surfaces tend to be reduced, particularly in the posterior branchial arches. Traditionally, this purported adaptation to living in hypoxic water has been suggested to prevent oxygen taken up from air in the air breathing organ from being immediately lost across the gills into

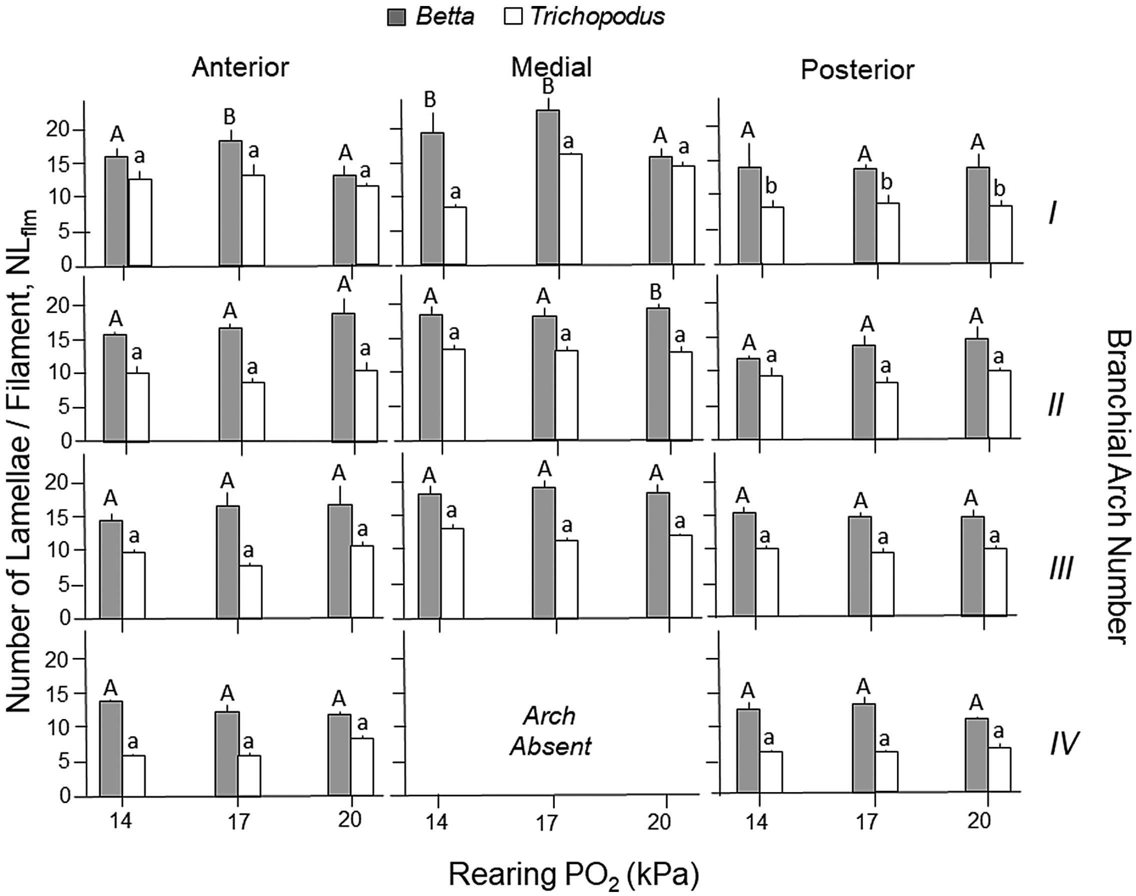

FIGURE 9 Number of lamellae per filament per arch (NL_{flm}) at the onset of air-breathing as a function of rearing PO_2 in *Betta splendens* (38 dpf) and *Trichopodus trichopterus* (35 dpf). Means and positive standard errors are presented. Letters (capital for *B. splendens*, small for *T. trichopterus*) indicate intraspecific statistics of differences of means, corrected for body length. n = 9 for *B. splendens*, n = 9 for *T. trichopterus*

hypoxia water (Burggren, 1979; Burggren, Johansen, & McMahon, 1986; Graham, 1997; Randall et al., 1981). Thus, it is possible that reduced branchial surface area in larval *B. splendens* reared in hypoxia does not reflect an inability to expand their gill size, but rather an adaptive response to aquatic hypoxia that minimizes oxygen loss to the water once air breathing begins in the developing larva. This time-honored argument hinges in part on the blood oxygen affinity of the two species. *Betta splendens* has an oxygen affinity at a pH of 7.6 of <0.5 kPa, compared to ~2 kPa in *T. trichopterus* (Mendez-Sanchez & Burggren, in press). Despite these differences, both species nonetheless have a blood P_{50} that appears sufficiently high to saturate arterial blood at the levels of hypoxia used in this study, even when considering admixture of oxygenated venous blood from the labyrinth mixing with systemic venous blood prior to entering the ventricle.

Our results indicate that the decrease in TSA_{lam} could be compensated for an increase in TSA_{lab}, possibly in preparation for a future air-breathing way of life, as Blank (2009) suggested for older *T. trichopterus*.

In general, the branchial morphometric measurements did not show differential responses related to PO_2 treatments within species. In contrast, the differences between the two fish species are statistically significant in most of the gill morphometrics, with *B. splendens* tending to have larger gills at equivalent developmental stages compared to the *T. trichopterus*.

4.2.3 | Labyrinth responses to chronic hypoxia

Larval *B. splendens* larvae reared in hypoxia (17 kPa) for 35 days showed a significant increase of 30% in the total surface of the labyrinth organ (TSA_{lab}) compared to control larvae, when correcting for body mass (Figure 11b). However, more severe hypoxia proved debilitating, inducing a significant 50% decrease in TSA_{lam} at 14 kPa. Clearly, the threshold for maintaining an adaptive stimulatory response of the labyrinth lies between 14 and 17 kPa.

The respiratory surfaces of *T. trichopterus* larvae did not show the same responses, but this might be explained by the fact that larval *B. splendens* were 2–3 times larger in both variables compared to *T. trichopterus* larvae.

4.3 | A species comparison—Influence of natural habitat on branchial and labyrinth morphology

We predicted that *B. splendens* would have different branchial features from *T. trichopterus* due to their different natural histories. The natural reproductive habitats of *B. splendens* are temporal isolated ponds or stagnant water of flood plains such as rice paddies. These habitats are frequently hypoxic, even anoxic on the bottom, because of the high temperatures and high organic content (Froese & Pauly, 2018; Monvises et al., 2009; Rainboth, 1996). Young larval *B. splendens* are not able to escape these hypoxic ecological conditions

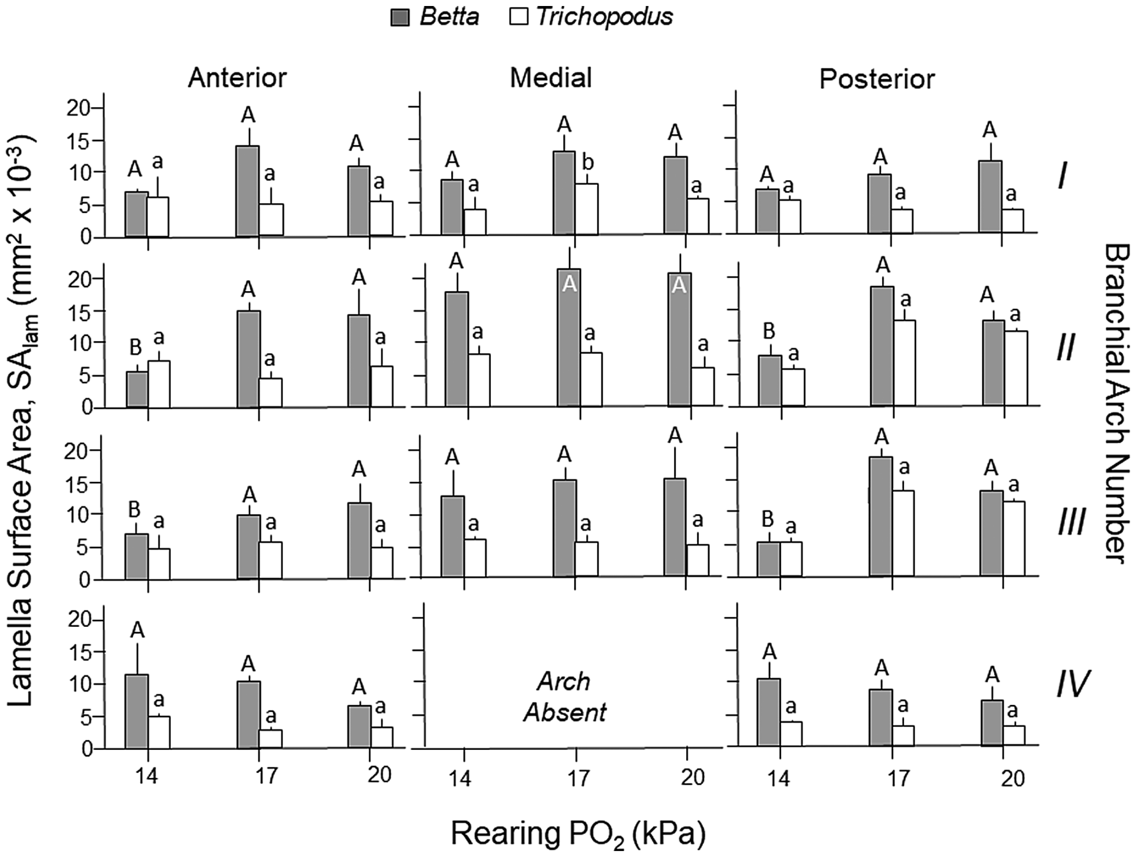

FIGURE 10 Area of individual lamella per filament per arch (SA_{lam}) at the onset of air-breathing as a function of rearing PO_2 in *Betta splendens* (38 dpf) and *Trichopodus trichopterus* (35 dpf). Means and positive standard errors are presented. Letters (capital for *B. splendens*, small for *T. trichopterus*) indicate intraspecific statistics of differences of means, corrected for body length. n = 9 for *B. splendens*, n = 9 for *T. trichopterus*

and natural selection may have led to a greater tolerance of aquatic hypoxia. In this situation we predicted that larval *B. splendens* could develop branchial features that facilitated its tolerance of aquatic hypoxia, such as a higher TSA_{lam}. Indeed, TSA_{lam} in *B. splendens* at the onset of air-breathing was five times larger than in *T. trichopterus* when corrected for interspecific body mass difference. This size relationship was manifested in all features of gill structure. *Betta splendens* also exhibited enhanced growth under mild hypoxic conditions. These features correlate well with the facultative air-breathing condition of *B. splendens* (Graham, 1997; Peters, 1978) and its ability to extract oxygen from the water at this larval stage (38 dpf).

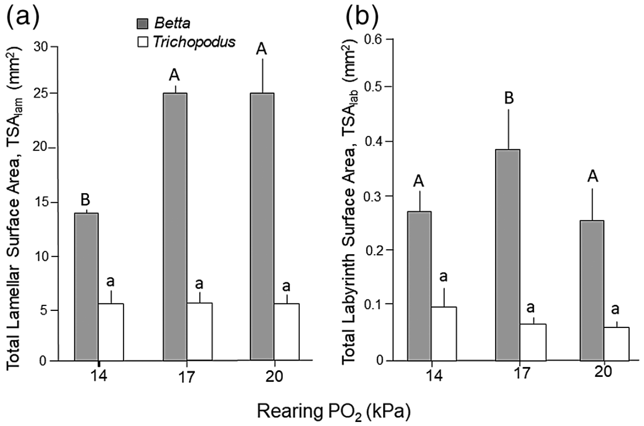

FIGURE 11 Respiratory surface areas at the onset of air-breathing as a function of rearing PO_2 in *B. splendens* (38 dpf) and *T. trichopterus* (35 dpf). (a) Lamellar respiratory surface (TSA_{lam}). (b) Labyrinth organ respiratory surface (TSA_{lab}) means and positive standard error are presented. Letters (capital for *B. splendens*, small for *T. trichopterus*) indicate intraspecific statistics of differences of means, corrected for body length. n = 9 for *B. splendens*, n = 9 for *T. trichopterus*

Trichopodus trichopterus, in contrast to *B. splendens*, occupies a natural reproductive habitat in lowland wetlands such as marshes, swamps and canals. This habitat experiences seasonal floods that facilitate lateral migrations from the mainstream, or other permanent water bodies, to flooded areas during the flood season and allows their return to the permanent water bodies at the onset of the dry season (Froese & Pauly, 2018; Rainboth, 1996). *Trichopodus trichopterus* is also a bubble nest builder, with male parental care (Ruber et al., 2006; Monvises et al., 2009). Compared to *B. splendens*, larval *T. trichopterus* at any age are more active swimmers (Mendez-Sanchez & Burggren, in press). This feature favors the ability to escape these open habitats and the hypoxia they present. Possibly for this reason, and in contrast to *B. splendens*, *T. trichopterus* did not exhibit developmental morphological plasticity in response to aquatic hypoxia in either growth rates or respiratory surfaces. Compared with larval *B. splendens*, larval *T. trichopterus* have relatively small respiratory surfaces. This shifts the dependence on gas exchange to the air breathing organ of this species, which also allows it to partially avoid aquatic hypoxia, and likely explains its obligate air-breathing condition (Blank, 2009; Blank & Burggren, 2014; Burggren, 1979; Graham, 1997; Herbert & Wells, 2001; Mendez-Sanchez & Burggren, 2014).

TABLE 1 Mass-specific total lamellar and labyrinth surface area at the onset of air-breathing in *Betta splendens* (day 38) and *Trichopodus trichopterus* (day 35). Mean values ±1 standard error are presented

Species	PO_2 (kPa)	n	Body length (mm)	Body mass (mg)	Total lamellar surface area (mm^2)	Total labyrinth surface area (mm^2)	Total mass-specific lamellar surface area (mm^2/mg)	Total mass-specific labyrinth surface area (mm^2/mg)
Betta splendens	20	5	0.19 ± 0.53	14.23 ± 3.20	24.70 ± 3.30	0.25 ± 0.06	1.85 ± 0.33	0.018 ± 0.001
	17	5	0.26 ± 0.79	12.53 ± 2.86	24.89 ± 0.52	0.39 ± 0.07	2.18 ± 0.44	0.031 ± 0.001
	14	5	0.47 ± 0.78	10.93 ± 3.40	13.80 ± 0.17	0.28 ± 0.04	1.52 ± 0.44	0.028 ± 0.005
Trichopodus trichopterus	20	5	8.36 ± 0.19	4.27 ± 0.37	5.30 ± 0.87	0.06 ± 0.01	1.23 ± 0.13	0.014 ± 0.001
	17	5	8.23 ± 0.26	4.80 ± 0.27	5.41 ± 0.89	0.06 ± 0.01	1.12 ± 0.12	0.013 ± 0.001
	14	5	9.60 ± 0.47	7.37 ± 1.49	5.34 ± 1.25	0.10 ± 0.03	0.80 ± 0.21	0.013 ± 0.002

Finally, we note that the present study has not considered the cutaneous gas exchange surface of either species, which at least in *T. trichopterus* comprise a large proportion of the gas exchange in the earliest developmental phases (Blank & Burggren, 2014). Few studies have considered the developmental plasticity of the skin of developing vertebrates as influenced by hypoxia. In larval anuran amphibians, chronic hypoxic results in cutaneous remodeling that increases both the density of skin capillaries as well as the blood-water distance within the skin (Burggren & Mwalukoma, 1983).

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