Hypoxia-induced developmental plasticity of the gills and air-breathing organ of *Trichopodus trichopterus*

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The air-breathing blue gourami *Trichopodus trichopterus*, an anabantid with a suprabranchial labyrinth organ, was used to study morphological development of respiratory systems in response to chronic hypoxia (13% O_2, combined aquatic and aerial hypoxia). Overall growth (fork length, wet mass and cutaneous surface area) of *T. trichopterus* did not differ between control fish and those reared in hypoxia. Both lamellar and labyrinth surface areas of the hypoxic larvae, however, increased more rapidly than controls, producing *c*. 16% larger lamellar and 30% larger labyrinth mass-specific surface areas within the first 120 days of development. This is the first study to show developmental respiratory plasticity of a bimodally respiring fish. It reveals that chronic hypoxia stimulates development of the gills and air-breathing organ, and that labyrinth growth is even more sensitive to hypoxia than branchial growth.

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Key words: bimodal gas exchange; branchial hypertrophy; labyrinth; larval development.

INTRODUCTION

Aquatic hypoxia, a common occurrence in many freshwater habitats, has long been viewed as a key driver for the evolution of air breathing in vertebrates (Carter, 1957; Johansen, 1970; Randall *et al.*, 1981; Little, 1983; Graham, 1997). Importantly, selection acts on every life-history stage, not just adults, so it is critical to understand how hypoxia shapes developing animals as well as mature forms (Burggren, 1992). Yet, despite the significance placed on aquatic hypoxia as a driving force in the evolution of air breathing, no study has comprehensively examined the developmental effects of hypoxia on respiratory ontogeny of air-breathing fishes.

Hypertrophy of organs and systems involved in oxygen acquisition and transport may occur when animals are reared in hypoxia (Loudon, 1989; Spicer & El-Gamal, 1999; Chapman *et al.*, 2002; Henry & Harrison, 2004) and as such comprises a classic example of developmental plasticity. The effects of hypoxic exposure on the general morphology of developing aquatic fishes are inconsistent, probably because varying levels of hypoxia and duration of exposure, as well as inter and intraspecific variation, play a large role in the overall development of an animal in response to hypoxia. For example, Atlantic cod *Gadus morhua* L.1758 (Chabot & Dutil, 1999)

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and turbot *Scophthalmus maximus* (L. 1758) (Pichavant *et al.*, 2000) reared in *c*. 10% O_2 exhibited decreased growth rates, but no effects were observed in Atlantic menhaden *Brevoortia tyrannus* (Latrobe 1802) and spot *Leiostomus xanthurus* Lacépède 1802 at even more severe ($<5\%$ O_2) hypoxic rearing levels (McNatt & Rice, 2004). The African cichlid *Pseudocrenilabrus multicolor victoriae* Seegers 1990, developing in hypoxic (*c*. 3% O_2) water, had larger respiratory surface areas, *i.e.* increased gill filament length and lamellar surface area, than those that developed in normoxia (Chapman *et al.*, 2000). Arctic charr *Salvelinus alpinus* (L. 1758) reared in hypoxia (*c*. 5% O_2) for 47 days post-hatch showed no difference in overall gill surface area (McDonald & McMahon, 1977).

The studies cited above showing considerable and sometimes conflicting interspecific variation were conducted on strictly water-breathing fishes. No study, to date, has observed the respiratory effects of rearing an air-breathing fish in hypoxia, even though such studies would be useful in understanding how hypoxia acts as a profound selection pressure in the evolution of air breathing. The purpose of this study was to observe morphological plasticity of an air-breathing fish, the blue gourami *Trichopodus trichopterus* (Pallas 1770), reared in chronic moderate hypoxia, with particular emphasis on the development of respiratory structures. It was hypothesized that the respiratory structures of developing *T. trichopterus* would show differential rates of growth, with those of the hypoxic group elevated over those of the normoxic group.

Trichopodus trichopterus is in the suborder Anabantoidei, consisting of five families native to south-east Asia (Das, 1927; Peters, 1978; Graham, 1997). Fishes of this suborder are referred to as labyrinth fishes because the air-breathing organ is a labyrinth-like structure located in paired suprabranchial chambers, residing behind the opercula and above the gills (Das, 1927; Bader, 1937; Peters, 1978). Adult *T. trichopterus* are continuous, obligatory air breathers (Burggren, 1979; Graham, 1997), but details of the developmental progression towards this lifestyle have not been previously determined. Embryos and young larvae of fishes typically are entirely aquatic and rely upon diffusion alone to meet their oxygen requirements (Rombough, 2007). As body size increases, blood convection begins to contribute to oxygen distribution, and the later development of the gills leads to active oxygen uptake *via* branchial respiration. In air-breathing species, the transition to air breathing commences when the diffusion distances at the skin and gills increase to the point when oxygen uptake across these surfaces is no longer adequate (Prasad, 1988; Graham, 1997). For anabantids, the onset of air breathing usually takes place at $18-20$ days post-fertilization (dpf), when the animal has reached a fork length (L_F) of $10-12$ mm (Das, 1927; Peters, 1978; Graham, 1997). *Trichopodus trichopterus* thus serves as an excellent model for investigating developmental plasticity as it relates to bimodal breathing.

MATERIALS AND METHODS

MAINTENANCE OF ADULT FISH

Adult *T. trichopterus* were purchased from local retailers and held in 38 l glass aquariums, with three to five adults per tank, segregated by sex. Water was maintained at $27°$ C, range $\pm 1°$ C, and biweekly water tests were conducted to ensure that pH, nitrite, nitrate and ammonia

levels were kept within an appropriate range (Degani & Schreibman, 1993; Linke, 1994). Adults were routinely fed Tetramin (www.tetra-fish.com) dry fish flakes once daily. To be conditioned for breeding, feedings were increased to twice daily and included Tetramin flakes, fresh and frozen brine shrimp *Artemia* sp. and frozen bloodworms *Chironomus* sp. After 2 weeks of conditioning, a pair of adults was placed in a separate 38 l breeding tank. Following successful breeding, both adults were removed from the breeding tank and replaced into their respective holding tanks. Adult stocks were replaced every 12–18 months, and breeding took place every 1–2 months.

REARING OF EMBRYOS

Embryos were collected from breeding tanks <12 h dpf and were randomly placed in one of the two 18 l glass aquaria filled with 3 l water at 27·0° C (Hisaoka & Firlit, 1962). Each tank was enclosed in a larger (38 l) sealed glass tank to maintain high humidity in the air above the water, suggested as beneficial for larvae as they change to air breathing (Alderton, 2008). Atmospheric air was bubbled through the water of the control tank to maintain normoxia (20·9% O_2, range ± 0·1% O_2). For hypoxic exposure, an air pump in the 38 l tank bubbled 13% O_2 through the water to create a controlled level of aquatic hypoxia. The oxygen level of the water of each rearing group was confirmed several times each week using an oxygen microelectrode (Model 16-730, Microelectrodes, Inc.; www.microelectrodes.com). Nitrogen gas, as regulated by a ProOx oxygen controller (BioSpherix; www.biospherix.com) calibrated weekly, was introduced into the space above the water of the experimental tank to achieve a moderate level of aerial hypoxia (13·0% O_2, range ±0·1% O_2). Very young *T. trichopterus* larvae are buoyant and frequently occupy the top strata of the water column (Hisaoka & Firlit, 1962; Graham, 1997), so both available respiratory phases (air and water) were made hypoxic to ensure a fixed, known level of hypoxic exposure.

The hypoxia level used for these experiments was selected based on preliminary studies of *T. trichopterus* larvae, suggesting that early chronic exposure to a more severe hypoxia of 10% O_2 resulted in visible morphological defects and increased mortality (T. Blank & F. Mendez-Sanchez, unpubl. data) and that less severe levels of chronic hypoxia (15% O_2) did not induce significant alterations of observed physiological variables, *i.e.* heart rate and gill ventilation rate.

Larvae were fed a mixture of dry Tetramin fry food, Cyclop-Eeze (www.cyclop-eeze.com) and *Spirulina* sp. three times daily until *c.* 2 weeks of age, at which time they were large enough to consume freshly hatched *Artemia* sp. fed twice daily from then on. Tank waste was syphoned daily, and additional water was syphoned and replaced twice each week or as conditions necessitated, as indicated by biweekly water tests of pH, nitrite, nitrate and ammonia levels. The water level of the larval tank was increased throughout development to avoid high densities that may have compromised larval growth.

WHOLE-BODY MEASUREMENTS

From fertilization to 135 dpf, developing *T. trichopterus* from both rearing groups were randomly selected on a daily (1–14 dpf) to weekly (15–120 dpf) basis. Following 18–24 h food deprivation, *T. trichopterus* were euthanized by placement in a solution of >250 mg l^{-1} of MS-222. Images from light microscopy (Nikon Eclipse E200 compound microscope; www.nikoninstruments.com) captured *via* a Javelin digital camera and analysed with ImagePro (Media Cybernetics; www.mediacy.com) software were employed to measure L_F (±0·1 mm) of *T. trichopterus* larvae (1–14 dpf). The L_F of juveniles (15–120 dpf) was measured with a calliper from snout to fork of tail. After recording L_F, larvae and juveniles were placed on pre-weighed squares of aluminium foil, blotted dry and total body wet mass (M_{TB}; ±0·1 mg) was obtained. Due to their small size, younger larvae (1–14 dpf) were weighed in groups, with 10–20 animals per group, and the average larval M_{TB} was calculated. Older larvae and juveniles (15–120 dpf) were weighed individually. Juveniles, with a dorso-ventrally flattened body form, were traced twice, once on each side of the

body, and outlines were measured with ImagePro software to estimate cutaneous surface area (± 1 mm^2).

RESPIRATORY SURFACE AREA MEASUREMENTS

Larvae and juveniles were assessed for respiratory measurements from days 40 to 120. Animals to be dissected for morphological observations of the gills and labyrinth organ were also euthanized as described above, and measurements of L_F, M_{TB} and cutaneous surface area were recorded. Animals were then placed in 10% neutral buffered formalin for 18–24 h to sufficiently harden the specimen in preparation for dissection, while minimizing tissue shrinkage. After formalin treatment, the opercula were removed, and gills and labyrinth organs were carefully extracted and immersed in 1% alcian blue for 5 s to allow for easier microscopic observation of respiratory structures. Measurement of gill morphology followed that of Hughes (1966, 1984) and included gill arch (GA) lengths, selected filament lengths, number of filaments per arch, number of secondary lamellae per selected filament and surface area of secondary lamellae. Each measurement was made for the gills on both left and right sides of the body, and the final recorded measurement represents an average of the two sides.

All morphological measurements of the gills were obtained using ImagePro image analysis software. Filaments and secondary lamellae were quantified by dividing each GA into thirds according to the number of filaments present. The central filament of each third was then selected for measurements of filament length, number of secondary lamellae present and single lamellar surface area. Filament density was calculated as number of filaments per GA length, while lamellar density was calculated as the number of secondary lamellae per filament length. For each GA, surface area estimations of single secondary lamellae were obtained by multiplying the length and width of several lamellae of selected filaments, and then doubling to account for the top and bottom of the lamella. Lamellar surface area of each GA was calculated by determining the lamellar surface area of single lamellae and multiplying by the lamellar density of the corresponding filament, and again by the filament density of the corresponding GA. Lamellar surface area was then summed for all four GAs, and this value doubled to account for the gills on both sides of the fish, yielding total lamellar surface area.

In the early stages of development, the labyrinth is a relatively simple, plate-like organ. To calculate labyrinth surface area (mm^2), microscopic images of the labyrinth were taken from three different angles, and the outer circumference of the organ was traced with ImagePro software. The average circumference recorded for each labyrinth organ was used to calculate the surface area of one side of the labyrinth. This value was then doubled to account for both sides of the labyrinth and was again doubled to account for left and right labyrinth organs, yielding the total labyrinth surface area for each fish observed.

Total respiratory surface area was estimated by summing cutaneous, total lamellar and labyrinth surface areas.

STATISTICAL ANALYSES

Trichopodus trichopterus body size and the relative growth of respiratory organs become highly variable as development progresses. Thus, rather than comparing morphometric development of respiratory structures on the basis of chronological age, measurements were typically expressed relative to M_{TB}.

Data were analysed for significant differences between rearing groups and throughout development (dpf) within each group using two-way analysis of variance (ANOVA). If a significant interaction between rearing group and development was found, a *post hoc* test (Holm–Sidak multiple comparisons procedure) was employed. Regression analysis was employed when data were plotted against M_{TB}, and *t*-tests were utilized to compare the slopes of regression lines between groups. Values of respiratory surface area by structure, analysed as a percentile of total respiratory surface area, were first arc-sine transformed before performing appropriate statistical tests. All statistical tests adopted a significance level of $P < 0.05$ and were analysed with Sigma Stat (Systat; www.systat.com) software. All statistics of morphological development are summarized in Tables I–V.

RESULTS

WHOLE-BODY MEASUREMENTS

As expected, L_F increased significantly ($P < 0.001$) in a linear fashion from c. 3 mm at hatching to c. 30 mm by 120 dpf, in both normoxic and hypoxic rearing groups. There was no significant difference in growth rate between groups [Table I and Fig. 1(a)]. The M_{TB} also increased significantly ($P < 0.001$) from c. 0.1 mg at hatching to c. 250 mg by 120 dpf and was best described by a polynomial (quadratic) equation, again with no significant difference between normoxic and hypoxic populations [Table I and Fig. 1(b)]. Finally, cutaneous surface area significantly increased c. five-fold over the M_{TB} studied [Table I and Fig. 1(c)], again with no significant difference between rearing groups.

GILL DEVELOPMENT

Considerable differences existed between GAs with respect to length, filament numbers, filament lengths and lamellar surface area as follows: GA-III > GA-IV > GA-V > GA-VI [Fig. 2(a)]. Despite these size differences, all arches grew in proportion during development in normoxia and hypoxia.

Total lamellar surface area significantly ($P < 0.001$) increased with M_{TB} for both rearing groups [Table II and Fig. 3(a)]. The slope describing the hypoxic group was significantly ($P < 0.001$) greater than that of the normoxic group. When total lamellar surface area was considered as a mass-specific measurement (mm^2 mg^{-1} M_{TB}), the hypoxic group had a significantly ($P < 0.05$) higher value, revealing c. 16% greater mass-specific lamellar surface area than the normoxic group over the observed developmental period [Table V and Fig. 4(a)].

Individual components of gill morphology that contributed to the overall increase in lamellar surface area also showed significant differences. Lamellar surface area per GA significantly increased in all arches for both rearing groups, with the exception of GA-VI of the normoxic group (Table II). The greatest increase in lamellar surface area was observed in GA-III of the hypoxic group (c. 10-fold). The slope of the hypoxic regression was significantly greater ($P < 0.01$) than that of the normoxic group at all GAs. Single lamellar surface area increased significantly with M_{TB} at

TABLE I. Summary statistics of whole-body measurements of *Trichopodus trichopterus* reared in normoxia (mean ± S.E. total body mass, $M_{TB} = 28 ± 4$ mg) or hypoxia (mean ± S.E. $M_{TB} = 39 ± 8$ mg) to 135 days post-fertilization (dpf)

Rearing group	L_T (mm)	M_{TB} (mg)	A_{CS} (mm^2)
Normoxia	$L_F = 1.74 + 0.20T$, $n = 333$, $r^2 = 0.78$, $P < 0.001$	$M_{TB} = 3.27 - 0.72\,T + 0.03\,T^2$, $n = 215$, $r^2 = 0.47$, $P < 0.001$	$A_{CS} = -18.1 + 2.04\,T$, $n = 16$, $r^2 = 0.43$, $P < 0.01$
Hypoxia	$L_F = 1.44 + 0.22\,T$, $n = 90$, $r^2 = 0.78$, $P < 0.001$	$M_{TB} = 8.40 - 1.04\,T + 0.03\,T^2$, $n = 80$, $r^2 = 0.57$, $P < 0.001$	$A_{CS} = -93.1 + 3.36\,T$, $n = 10$, $r^2 = 0.54$, $P < 0.05$
Normoxia v. hypoxia	NS	NS	NS

NS, not significantly different ($P > 0.05$); L_F, fork length; A_{CS}, cutaneous surface area; T, time (dpf).

TABLE II. Summary statistics of branchial surface area and lamellar characteristics of *Trichopodus trichopterus* reared in normoxia or hypoxia to 135 days post-fertilization (dpf). Mean ± S.E. normoxic total body mass $M_{TB} = 94 \pm 15$ mg and hypoxic mean ± S.E. $M_{TB} = 89 \pm 24$ mg. $P < 0.01$ unless otherwise indicated

Rearing group	Gill arch	Total lamellar surface area (mm^{-2})	Single lamellar surface area (mm^{-2})	Lamellar count	Lamellar density (secondary lamellae mm^{-1} filament length)
Normoxia	III	$y = 0.93 + 0.09\,M_{TB}$, $n = 13$, $r^2 = 0.91$	$y = 0.004 + 0.000\,M_{TB}$, $n = 12$, $r^2 = 0.91$	$y = 616.0 + 7.3\,M_{TB}$, $n = 18$ $r^2 = 0.92$	$y = 69.0 + 2.2\,M_{TB}$, $n = 18$ NS
	IV	$y = 1.59 + 0.06\,M_{TB}$, $n = 13$, $r^2 = 0.90$	$y = 0.005 + 0.000\,M_{TB}$, $n = 12$, $r^2 = 0.82$	$y = 496.0 + 4.9\,M_{B}$, $n = 18$, $r^2 = 0.90$	$y = 70.0 - 0.1\,M_{TB}$, $n = 18$, $r^2 = 0.37$
	V	$y = 0.96 + 0.02\,M_{TB}$, $n = 13$, $r^2 = 0.85$	$y = 0.004 + 0.000\,M_{TB}$, $n = 12$, $r^2 = 0.76$	$y = 285.0 + 2.7\,M_{TB}$, $n = 18$, $r^2 = 0.87$	$y = 64.0 + 1.5\,M_{TB}$, $n = 18$, NS
	VI	$y = 0.63 + 0.19\,M_{TB}$, $n = 5$, NS	$y = 0.002 + 0.000\,M_{TB}$, $n = 5$, $r^2 = 0.83$	$y = 193.0 \pm 32\,M_{TB}$, $n = 5$, NS	$y = 57.0 + 3.9\,M_{TB}$, $n = 5$, NS
	Total	$y = 4.5 \pm 0.34\,M_{TB}$, $n = 13$, $r^2 = 0.97$		$y = 2887.0 \pm 29\,M_{TB}$, $n = 18$, $r^2 = 0.93$	
Hypoxia	III	$y = -0.64 + 0.12\,M_{TB}$, $n = 7$, $r^2 = 0.95$	$y = 0.003 + 0.000\,M_{TB}$, $n = 7$, $r^2 = 0.93$	$y = 621.0 + 6.9\,M_{TB}$, $n = 9$, $r^2 = 0.88$	$y = 68.0 - 0.06\,M_{TB}$, $n = 9$, $r^2 = 0.66$
	IV	$y = 1.86 + 0.07\,M_{TB}$, $n = 7$, $r^2 = 0.93$	$y = 0.006 + 0.000\,M_{TB}$, $n = 7$, $r^2 = 0.85$	$y = 475.0 + 5.2\,M_{TB}$, $n = 9$, $r^2 = 0.90$	$y = 59.0 - 2.9\,M_{TB}$, $n = 9$, NS
	V	$y = 0.61 + 0.02\,M_{TB}$, $n = 7$, $r^2 = 0.96$	$y = 0.003 + 0.000\,M_{TB}$, $n = 7$, $r^2 = 0.96$	$y = 287.0 + 2.4\,M_{TB}$, $n = 9$, $r^2 = 0.88$	$y = 65.0 - 0.06\,M_{TB}$, $n = 9$, $r^2 = 0.67$
	VI	$y = 0.30 + 0.01\,M_{TB}$, $n = 7$, $r^2 = 0.81$	$y = 0.003 + 0.000\,M_{TB}$, $n = 5$, NS	$y = 94.0 + 1.9\,M_{TB}$, $n = 7$, $r^2 = 0.88$	$y = 66.0 - 1.7\,M_{TB}$, $n = 7$, NS
	Total	$y = 5.00 + 0.42\,M_{TB}$, $n = 7$, $r^2 = 0.98$		$Y = 2930 + 33\,M_{TB}$, $n = 7$, $r^2 = 0.92$	
Normoxia *v.* hypoxia	III IV V VI Total	$P < 0.001$, $P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.001$	$P < 0.001$, NS, $P < 0.001$, $P < 0.001$,	NS, NS, NS, $P < 0\,0.01$, NS	NS, NS, NS, NS,

NS, not significantly different ($P > 0.05$).

TABLE III. Summary statistics of branchial filament characteristics of *Trichopodus trichopterus* reared in normoxia or hypoxia to 135 days post-fertilization (dpf). Mean ± S.E. normoxic total body mass M_{TB} = 94 ± 15 mg and mean ± S.E. hypoxic M_{TB} = 89 ± 24 mg. P < 0.01 unless otherwise indicated

Rearing group	Gill arch	Filament length (mm)	Filament count	Filament density (filaments mm^{-1})	Gill arch length (mm)
Normoxia	III	$y = 0.19+0.00\,M_{TB}$, $n = 18$, $r^2 = 0.84$	$y = 46.0+0.1\,M_{TB}$, $n = 18$, $r^2 = 0.89$	$y = 23.0 - 0.0\,M_{TB}$, $n = 18$, $r^2 = 0.38$	$y = 2.00+0.01\,M_{TB}$, $n = 18$, $r^2 = 0.93$
	IV	$y = 0.20+0.00^3\,M_{TB}$, $n = 18$, $r^2 = 0.84$	$y = 37.0+0.1\,M_{TB}$, $n = 18$, $r^2 = 0.83$	$y = 23.0 - 0.1\,M_{TB}$, $n = 18$, $r^2 = 0.23$	$y = 1.50+0.01\,M_{TB}$, $n = 18$, $r^2 = 0.94$
	V	$y = 0.15+0.00\,M_{TB}$, $n = 18$, $r^2 = 0.70$	$y = 31.0+0.1\,M_{TB}$, $n = 18$, $r^2 = 0.77$	$y = 24.0 - 0.5\,M_{TB}$, $n = 18$, NS	$y = 1.20+0.001\,M_{TB}$, $n = 18$, $r^2 = 0.90$
	VI	$y = 0.11+0.01\,M_{TB}$, $n = 7$, NS	$y = 32.0+1.4\,M_{TB}$, $n = 9$, NS	$y = 25.0 - 0.0\,M_{TB}$, $n = 7$, $r^2 = 0.58$, $P < 0.05$	$y = 1.10+0.00\,M_{TB}$, $n = 13$, $r^2 = 0.77$
	Total		$y = 300.0+0.8\,M_{TB}$, $n = 8$, $r^2 = 0.98$		
Hypoxia	III	$y = 0.20+0.00\,M_{TB}$, $n = 10$, $r^2 = 0.89$	$y = 45.0+0.1\,M_{TB}$, $n = 11$, $r^2 = 0.81$	$y = 23.0 - 0.0\,M_{TB}$, $n = 11$, $r^2 = 0.57$	$y = 1.90+0.01\,M_{TB}$, $n = 11$, $r^2 = 0.89$
	IV	$y = 0.20+0.00\,M_{TB}$, $n = 10$, $r^2 = 0.86$	$y = 37.0+0.1\,M_{TB}$, $n = 11$, $r^2 = 0.86$	$y = 24.0 - 0.0\,M_{TB}$, $n = 11$, $r^2 = 0.55$	$y = 1.50+0.01\,M_{TB}$, $n = 11$, $r^2 = 0.91$
	V	$y = 0.14+0.01\,M_{TB}$, $n = 10$, $r^2 = 0.87$	$y = 31.0+0.1\,M_{TB}$, $n = 11$, $r^2 = 0.87$	$y = 25.0 - 0.6\,M_{TB}$, $n = 11$, NS	$y = 1.20+0.01\,M_{TB}$, $n = 11$, $r^2 = 0.94$
	VI	$y = 0.07+0.00\,M_{TB}$, $n = 8$, $r^2 = 0.76$	$y = 24.0+0.1\,M_{TB}$, $n = 9$, $r^2 = 0.79$	$y = 24.0 - 0.9\,M_{TB}$, $n = 8$, NS	$y = 0.80+0.00\,M_{TB}$, $n = 10$, $r^2 = 0.90$
	Total		$y = 261.0+0.9\,M_{TB}$, $n = 9$, $r^2 = 0.84$		
Normoxia *v.* hypoxia	III IV V VI Total	$P < 0.05$, $P < 0.001$, $P < 0.001$, $P < 0.001$	NS, NS, NS, NS, NS	NS, NS, NS, NS	NS, $P < 0.01$, NS, $P < 0.01$

NS, not significantly different ($P > 0.05$).

TABLE IV. Summary statistics of labyrinth surface area and total respiratory surface area of *Trichopodus trichopterus* reared in normoxia or hypoxia to 135 days post-fertilization (dpf). Mean $\pm$ S.E. normoxic total body mass $M_{TB} = 94 \pm 15$ mg and mean $\pm$ S.E. hypoxic $M_{TB} = 89 \pm 24$ mg

Rearing group	Total labyrinth surface area (mm^2)	Total respiratory surface area (mm^2)
Normoxia	$y = -0.66 + 0.02\,M_{TB}$, $n = 20,\ r^2 = 0.97$, $P < 0.001$	$y = 39.70 + 1.21\,M_{TB}$, $n = 10,\ r^2 = 0.99$, $P < 0.001$
Hypoxia	$y = -0.34 + 0.03\,M_{TB}$, $n = 11,\ r^2 = 0.98$, $P < 0.001$	$y = 39.30 + 1.21\,M_{TB}$, $n = 7,\ r^2 = 0.98$, $P < 0.001$
Normoxia *v.* hypoxia	$P < 0.01$,	NS

NS, not significantly different ($P > 0.05$).

all GAs of both rearing groups, with the exception of GA-VI of the hypoxic group (Table II). The greatest increase in single lamellar surface area was observed in GA-IV of the hypoxic group (*c*. three-fold). The slopes for the hypoxic group were significantly greater than those of the normoxic group at GA-III, GA-V and GA-VI.

The total number of secondary lamellae per GA (lamellar count) significantly increased (*c*. three to four-fold) with M_{TB} at all GAs of both rearing groups, with the exception of GA-VI of the normoxic group (Table II). Thus, the slope of the hypoxic regression was significantly ($P < 0.001$) greater than that of the normoxic group at GA-VI. Total body lamellar count also significantly increased (*c*. three-fold) with M_{TB}, although there was no significant difference between rearing groups. Lamellar density showed a trend towards decreasing density as M_{TB} increased (Table II), but

TABLE V. Summary statistics of mass-specific lamellar, labyrinth and total respiratory surface areas of *Trichopodus trichopterus* reared in normoxia or hypoxia to 120 days post-fertilization (dpf). Mean $\pm$ S.E. normoxic total body mass $M_{TB} = 94 \pm 15$ mg and mean $\pm$ S.E. hypoxic $M_{TB} = 89 \pm 24$ mg

| Rearing group	Mass-specific total lamellar surface area (mm^2 mg^{-1})	Mass-specific total labyrinth surface area (mm^2 mg^{-1})	Mass-specific total respiratory surface area (mm^2 mg^{-1})
Normoxia	$y = 0.42 + 0.02\,M_{TB}$, $n = 13$, NS	$y = 0.01 + 0.00\,M_{TB}$, $n = 20,\ r^2 = 0.55$, $P < 0.001$	$y = 2.30 - 0.01\,M_{TB}$, $n = 11,\ r^2 = 0.89$, $P < 0.001$
Hypoxia	$y = 0.49 + 0.08\,M_{TB}$, $n = 7$, NS	$y = 0.02 + 0.00\,M_{TB}$, $n = 11,\ r^2 = 0.41$, $P < 0.05$	$y = 2.20 - 0.00\,M_{TB}$, $n = 7,\ r^2 = 0.59$, $P < 0.05$
Normoxia *v.* hypoxia	Slope: NS Elevation: $P < 0.05$	Slope: NS Elevation: $P < 0.01$	Slope: NS Elevation: NS

NS, not significantly different ($P > 0.05$).
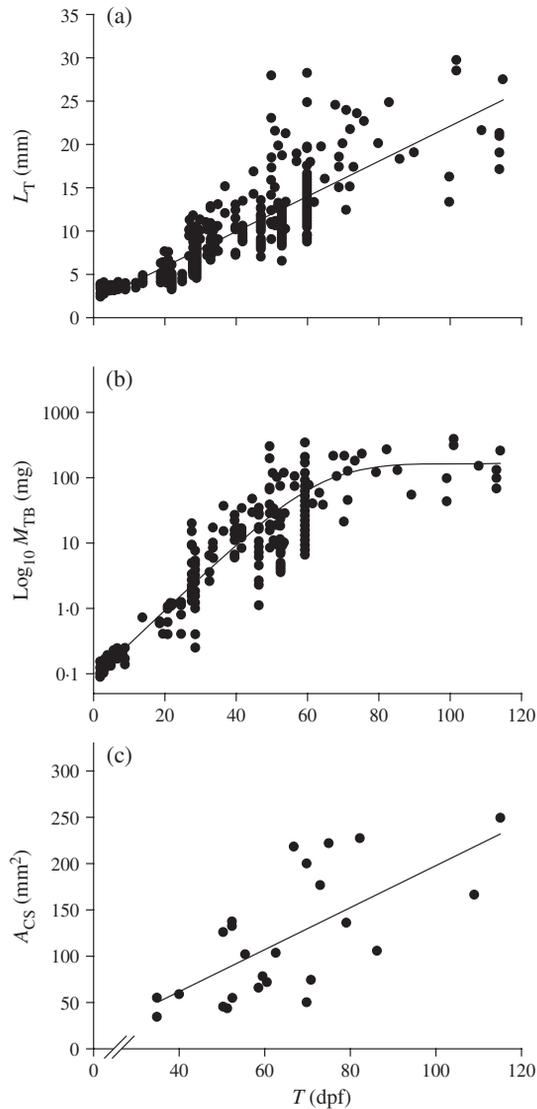
FIG. 1. Whole-body measurements as a function of development (time, T, to 120 days post-fertilization, dpf) of *Trichopodus trichopterus*. Normoxic and hypoxic populations showed no significant differences, and data were pooled for plotting. (a) Total body length (L_T), (b) $\log_{10}$ total body wet mass (M_{TB}) and (c) cutaneous surface area (A_{CS}) (see Table I).

significant differences were observed only in GA-IV of the normoxic group and in GA-III and GA-V of the hypoxic group.

The rate of filament growth (filament length) increased significantly with M_{TB} in all arches for both rearing groups, with the exception of GA-VI of the normoxic group (Table III). The greatest increases for both groups (*c*. two to three-fold) were observed in GA-III. The slopes of the regressions for the hypoxic group were significantly greater than those of the normoxic group at all GAs.
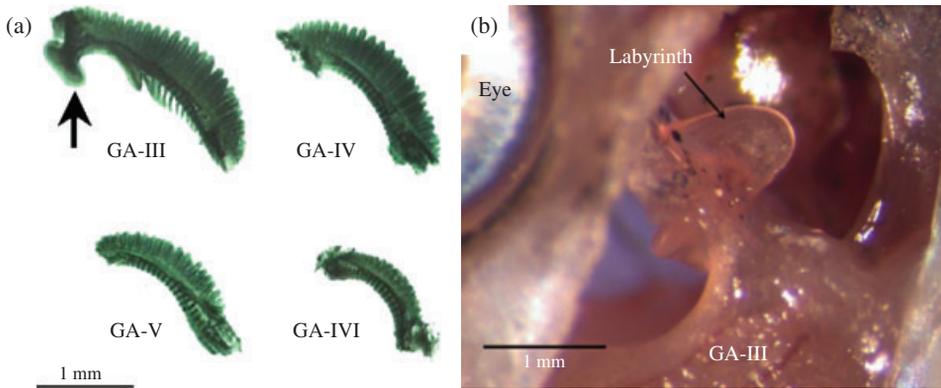
FIG. 2. Branchial structures of juvenile *Trichopodus trichopterus*. (a) Images of gill arches (GAs) III–VI of a juvenile *T. trichopterus* [35 days post-fertilization, dpf; total body length (L_T) = 13 mm; total body wet mass (M_{TB}) = 28 mg]. GA-III from which the labyrinth organ (⟶) arises and GA-IV are larger and have longer, better developed filaments and more secondary lamellae than GA-V and GA-VI. (b) Branchial dissection of juvenile *T. trichopterus* (60 dpf; L_T = 15 mm; M_{TB} = 40 mg), revealing labyrinth organ (⟶) and GA-III.

The number of filaments per GA increased significantly with M_{TB} at all arches for both rearing groups (c. 1·5-fold), with the exception of GA-VI of the normoxic group (Table III). There were no differences between slopes for any GA. Total body filament count also significantly increased with M_{TB}, and again no difference was found between rearing groups. Filament density decreased as M_{TB} increased (Table III), with significance observed in GA-III, GA-IV and GA-VI of the normoxic group and GA-III and GA-IV of the hypoxic group. No significant differences were found between the slopes of the rearing groups for any GA.

GA length increased significantly ($P < 0.001$) with M_{TB} for both normoxic and hypoxic rearing groups, approximately doubling for most arches over the developmental period observed (Table III). The slope of the regression for GA-VI was significantly greater in the hypoxic group, while that for GA-IV was significantly lower than in the normoxic group. The hypoxic group also revealed significantly lower elevations in GA-III, GA-IV and GA-V than the normoxic group.

LABYRINTH ORGAN DEVELOPMENT

The labyrinth organ begins development as a simple, plate-like growth from the base of GA-III (Fig. 2), before becoming increasingly elaborate and complex. Total labyrinth surface area increased significantly ($P < 0.001$), c. 20-fold M_{TB}, for both rearing groups [Table IV and Fig. 3(b)]. The rate of labyrinth growth, however, was significantly ($P < 0.01$) greater for the hypoxic group than the normoxic group. When total labyrinth surface area was calculated on a mass-specific basis, both rearing groups showed significant increases over the developmental period observed [Table V and Fig. 4(b)]. The hypoxic group, however, had a significantly ($P < 0.05$) larger increase, revealing c. 30% greater mass-specific labyrinth surface area than the normoxic group.
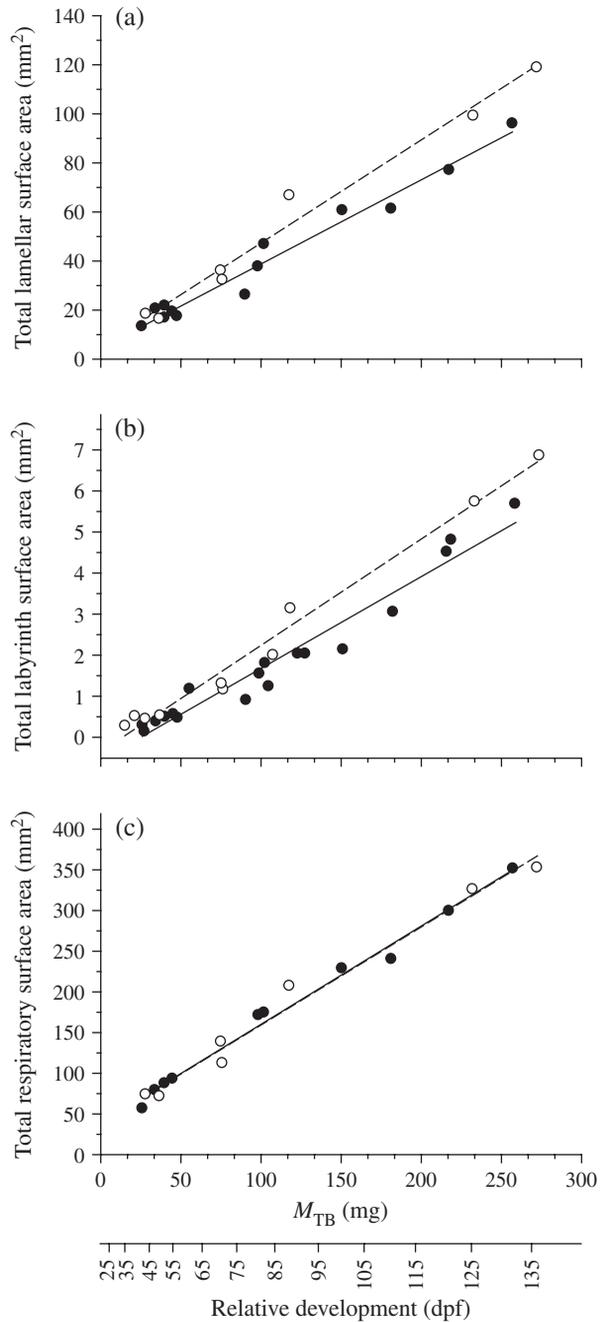
FIG. 3. Measurements of respiratory structures as a function of total body wet mass (M_{TB}) and relative devel-
opmental age (days post-fertilization, dpf) of *Trichopodus trichopterus* reared in normoxia (●, ——) or
hypoxia (○, – – –) to 135 dpf. (a) Total lamellar surface area, (b) total labyrinth surface area and (c) total
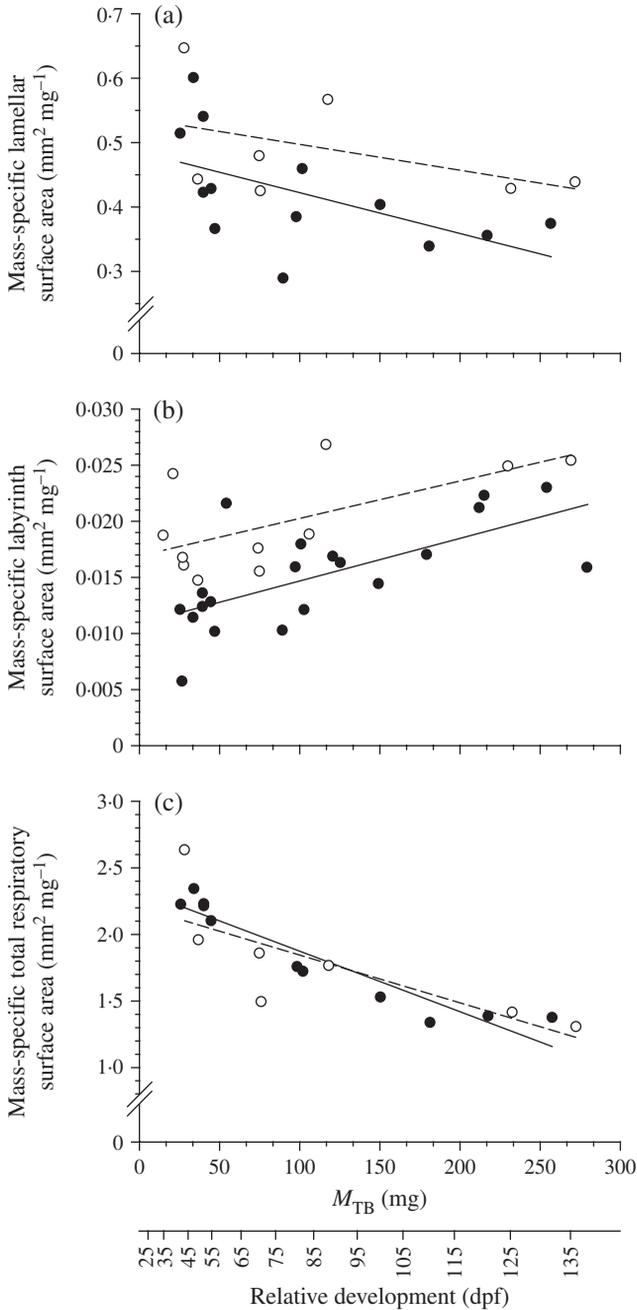respiratory surface area (cutaneous + lamellar + labyrinth) (see Tables II–IV).

FIG. 4. Mass-specific respiratory surface area by structure of *Trichopodus trichopterus* reared in normoxia (●, —) or hypoxia (○, – –) to135 days post-fertilization (dpf) as a function of total body wet mass (M_{TB}) and relative developmental age (dpf). (a) Mass-specific lamellar surface area, (b) mass-specific labyrinth surface area and (c) mass-specific total respiratory surface area (see Table V).
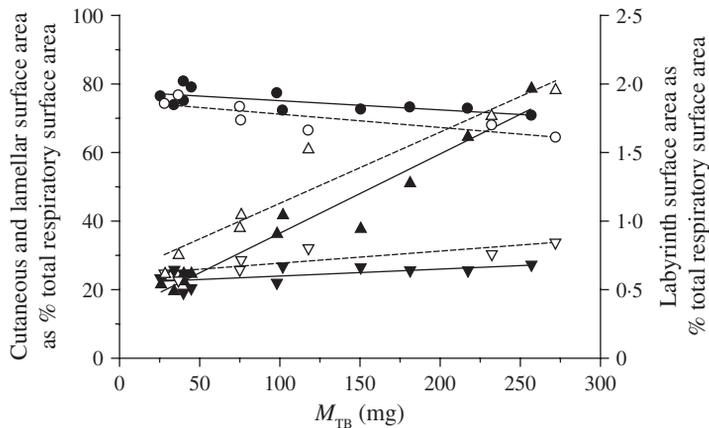
FIG. 5. Contribution to total respiratory surface area by each respiratory structure (relative surface area) of *Trichopodus trichopterus* reared in normoxia (●, ▲, ▼, ——) or hypoxia (○, △, ▽, - - - -) to 120 days post-fertilization (dpf). ○ represent the relative cutaneous surface area. △ represent the relative labyrinth surface area. ▽ represent the relative lamellar surface area.

TOTAL RESPIRATORY SURFACE AREA

Total respiratory surface area, calculated from the sum of cutaneous, lamellar and labyrinth surface areas, showed an expected significant ($P < 0.001$), nearly seven-fold increase over the measured growth period, with no significant difference between rearing groups [Table IV and Fig. 3(c)]. When total respiratory surface area was expressed as a mass-specific measurement, both groups showed significant declines over the developmental period observed, with no significant difference between rearing groups [Table V and Fig. 4(c)].

The surface area of each respiratory structure (skin, lamellae and labyrinth organs) was then considered as a percentile of total respiratory surface area (relative surface area; Fig. 5). The relative cutaneous and lamellar surface areas did not change for the normoxic group (c. 75 and 24%) over the developmental period studied. The relative cutaneous surface area of the hypoxic group significantly declined (c. 76 to 64%), while the relative lamellar surface area significantly increased (c. 23–34%). Relative labyrinth surface areas significantly ($P < 0.01$) increased for both the normoxic (c. 0.5–1.6%) and the hypoxic (c. 0.6–2.0%) groups.

DISCUSSION

WHOLE BODY SIZE AND SHAPE

The continual increase in L_F with larval development concurrent with the plateauing of M_{TB} at around 60 days suggests that juveniles have a lower condition factor K (Anderson & Neumann, 1996) than younger larvae. The M_{TB} and L_F indices are typically created to assess changes in body shape of adult fishes in varying nutritional states, not to assess the condition of developing fishes, whose growth involves genetically dictated changes in body shape. Development of an index applicable

during each stage of larval growth would be useful, but would probably have to be species-specific, given the different body shape trajectories during development by different fish species.

A number of aquatic teleosts respond to chronic hypoxia as larvae with significant declines in overall growth rates, but the specific response is dependent on the level and duration of hypoxia experienced. For example, larval *S. alpinus* exposed to chronic hypoxia (c. 5% O_2) for 47 days post-hatch weighed 20% less than their normoxic cohorts (McDonald & McMahon, 1977). The growth rate of *G. morhua* also declined when larvae were exposed to chronic hypoxia (c. 10% O_2), probably due to decreased food ingestion rates (Chabot & Dutil, 1999). Juvenile *B. tyrannus* and *L. xanthurus* showed similar reduced growth rates in extreme chronic hypoxia (c. 5% O_2), but growth was generally unaffected at lower levels of hypoxia (McNatt & Rice, 2004). On the other hand, chronic hypoxic exposure (c. 12%) in the zebrafish *Danio rerio* (Hamilton 1822) does not affect body mass and length until 60 days of development (Barrionuevo *et al.*, 2010). The lack of a significant effect of chronic hypoxia on L_F and M_{TB} in *T. trichopterus* in this study may be due to the less severe level of hypoxia employed compared to previous studies.

Cutaneous surface area expressed as g^{-1} body mass decreased with growth of the larvae, which simply reflects the relationship between changing surface area as volume of an object increases. For example, the cutaneous surface area of 1 day post-hatch walleye *Sander vitreus* (Mitchill 1818) accounts for >99% of the total respiratory surface area available but decreases to 58% by 200 g body mass because of both declining surface area to volume ratio and development of branchial structures (Rombough & Moroz, 1997). Cutaneous surface area in *T. trichopterus* of this study was unaffected by chronic hypoxia, although this was only measured for juvenile *T. trichopterus* (>30 dpf). It is possible that hypoxia-induced differences do exist in earlier stages, when cutaneous respiration plays a more important role in oxygen uptake (Brauner & Rombough, 2012). There is not the same degree of developmental plasticity of cutaneous surface area as there is for branchial or labyrinth surface areas, simply because a change in cutaneous surface area would of necessity require a change in body size and shape and may have a greater cost than benefit. This leaves open, however, that the vascularity of the skin might be modified by hypoxia in developing larval air-breathing fishes, as it is in developing larval bullfrogs *Lithobates catesbeiana* exposed to chronic hypoxia (Burggren & Mwalukoma, 1983). The physiological role of the skin in the overall gas exchange of developing air-breathing fishes, and how flexible this might be during larval growth, will be interesting to discern.

Also noteworthy is that this study found *T. trichopterus* to be slightly less sensitive to chronic hypoxia in early larval life than reported for this species in an independent study. Mendez-Sanchez & Burggren (2014) found that larvae experienced significant mortality below c. 15% chronic hypoxia, although mortality was reduced in the intermittent hypoxia group. Mendez-Sanchez & Burggren (2014) conducted experiments on survival and onset of air breathing using larvae from the same clutch, as preliminary studies had found significant intra-clutch variation for the onset of air breathing in the larval Siamese fighting fish *Betta splendens* Regan 1910. In contrast, this study used larvae from several different clutches and parents. In addition to the potential for intra-clutch variation, the susceptibility of larvae to hypoxia can reflect epigenetic influences related to maternal and paternal experiences (Ho & Burggren, 2010; Burggren, 2014).

GILL DEVELOPMENT

Lamellar gas exchange is influenced by a number of factors, including respiratory surface area, thickness of the blood–water diffusion barrier, oxygen capacity and binding affinity of haemoglobin and lamellar blood flow (McDonald & McMahon, 1977; Perry & McDonald, 1993; Evans *et al.*, 2005). Although the teleost gill is best known as a gas exchange organ, it also serves a number of other functions, including ion regulation, osmoregulation, acid–base balance and ammonia excretion (Evans *et al.*, 2005; Rombough, 2007). Ion exchange is a major function of the GAs and primary filaments of adult fishes (Laurent, 1984; Evans, 2008), and in developing animals, the gills may develop early to serve ion regulatory functions before they have a major role in respiration (Rombough, 2002, 2007; Brauner & Rombough, 2012). In the anabantid *Anabas testudineus* (Bloch 1792), GAs and filaments begin to differentiate by 24 h post-fertilization, but secondary lamellae do not develop until 4 dpf (Hughes *et al.*, 1986). Thus, GAs and filaments might also play a role in gas exchange until gills mature.

As with the *T. trichopterus*, GA-III and GA-IV in other anabantids are larger and more highly developed than GA-V and GA-VI (Das, 1927; Prasad, 1988; Maina, 2002), reflecting the greater importance of the more anteriorly positioned arches in aquatic gas exchange. Moreover, the efferent branchial arteries of GA-III and GA-IV supply blood to the labyrinth organ (Burggren, 1979; Munshi & Hughes, 1992; Graham, 1997) and are thus also integral to aerial respiration.

The decreased, rather than increased, growth rate of GA-IV of the hypoxic group was unexpected. The first two arches are generally presumed to be responsible for gas exchange, a notion supported by the increased lamellar surface area and filament length of these two arches in the hypoxic group. It may be that the more important role of these GAs in larval *T. trichopterus* is ion regulation, for example, and that the changes observed in the chronically hypoxic group were concomitant with an altered need for such function. In the pearl gourami *Trichopodus leeri* (Bleeker 1852), GA-III and GA-IV are responsible for ion regulation, as the number of mitochondria-rich cells increased in these arches upon exposure to ionic stress, while there was no difference in the number of mitochondria-rich cells of the more posterior GA-V and GA-VI (Huang *et al.*, 2007).

Anabantids have large-bore anterior-arterial shunts in GA-V and GA-VI (Olson *et al.*, 1986; Huang *et al.*, 2007), suggesting that these arches are specialized for the transport of oxygenated blood to the body rather than gas exchange *per se*. This is probably an adaptation to living in hypoxic environments, reducing the direct loss of oxygen across gills with a large respiratory surface area (Burggren, 1979; Munshi & Hughes, 1992). Thus, the findings of increased single and total lamellar surface areas and increased filament length in GA-V and GA-VI, and increased growth rate of GA-VI for the hypoxic group, were counterintuitive. As with the anterior GAs, this may be indicative of large non-respiratory roles of the posterior arches, filaments and lamellae. Alternatively, these arches may be more responsible for actual gas exchange and play less of a role as direct blood shunts, than was previously thought.

The general lack of significant differences between control and hypoxic groups in total lamellar count and density, and total filament count and density, suggests that the basic components of branchial structures for *T. trichopterus* are relatively fixed in development. The significant structural modifications in response to hypoxia in

T. trichopterus are at the gross anatomical level, namely in the gill shape, including length and surface area. The actual number and density of filaments and lamellae does not appear to contribute to the environmentally induced changes in branchial structure.

Secondary lamellae generally form on filaments around 4 dpf in anabantids (Hughes *et al.*, 1986; Prasad, 1988) and tremendously augment the surface area of the gills. This allows for improved branchial gas exchange as oxygen demand increases throughout development. Increasing surface area of the gills appears to be a common response by fishes to hypoxia. For example, single lamellar surface area of larval *S. alpinus* exposed to chronic hypoxia for 47 days post-hatch was 38% greater than the normoxic group (McDonald & McMahon, 1977), even though total lamellar surface area was unaffected due to the reduction of other gill parameters. For sea bass *Dicentrarchus labrax* (L. 1758) reared under different oxygen levels for 3 months, an inverse relationship was found between gill surface area and rearing oxygen partial pressure (PO_2) (Saroglia *et al.*, 2002). Total gill surface area (adjusted for mass) of *P. m. victoriae* reared for *c.* 5 months in hypoxia (*c.* 3% O_2) was *c.* 18% greater than that of fish reared in normoxia (Chapman *et al.*, 2000), which is similar to the *c.* 16% increase observed in mass-specific total lamellar surface area of the hypoxic group in this study. For the *T. trichopterus* reared in chronic hypoxia, increased total lamellar surface area indicates that the gills are plastic in their initial development. Yet, it also suggests that this species continues to invest energy in processes that maximize oxygen uptake *via* branchial respiration, even after air breathing has commenced, which typically occurs between days 30 and 36 (Mendez-Sanchez & Burggren, 2014).

The thickness of the blood–water diffusion distance for *T. trichopterus* was not measured in this study. Hypoxia reduces the blood–water diffusion distance in some adult teleosts (Sollid & Nilsson, 2006; Matey *et al.*, 2008) and warrants further investigation in anabantids.

LABYRINTH ORGAN DEVELOPMENT

The first traces of the labyrinth organ in *A. testudineus* and *Macropodus opercularis* (L. 1758) are not present until larvae reached a length of 12 mm (Das, 1927), and even in 6 month-old (1 g) *A. testudineus*, the labyrinth organ is 'not well differentiated, having only one saucer-shaped plate' (Munshi & Hughes, 1986). In this study, the smallest fish observed in both rearing groups (*c.* 20 mg) had little more than a labyrinth bud (*c.* 0·3 mm^2), and the labyrinth organ remained a relatively simple structure throughout the developmental period observed, consisting of a single vascularized plate. While the surface area of the labyrinth organ increased >20-fold over the developmental period studied, the contribution of the labyrinth to total respiratory surface area remained relatively small. It is possible that the labyrinth organ, in association with the highly vascularized surrounding suprabranchial chamber, plays a role in aquatic respiration prior to the complete transition to air breathing. Thus, the observed differential growth of the labyrinth organ may be in response to the different aquatic oxygen levels. Bader (1937) found that lack of access to air precludes differentiation of the air-breathing organ in developing *M. opercularis* and hypothesized that some factor involved in aerial respiration acts as a necessary environmental stimulus for differentiation of the labyrinth organ and suprabranchial

chambers. As even the smallest *T. trichopterus* used for branchial dissections were already actively taking air into their suprabranchial chambers, further studies are needed to determine the relative importance of aerial *v.* aquatic stimuli for labyrinth organ development.

TOTAL RESPIRATORY SURFACE AREA

Significant increases in total lamellar and labyrinth surface areas occurred in *T. trichopterus* reared in hypoxia. Yet, there was no difference in total respiratory surface area between normoxic and hypoxic rearing groups. This can be explained by taking into consideration the very large contribution of cutaneous surface area to total respiratory surface area (and the concomitant smaller contributions of lamellar and labyrinth surface areas) in young *T. trichopterus*. The slight, albeit insignificant, reduction of cutaneous surface area in the hypoxic group probably countered the significant increases in total lamellar and labyrinth surface areas. Additionally, the volume of the suprabranchial chamber was not taken into consideration in this study and may have possibly altered total respiratory surface area between groups.

In conclusion, larvae of the *T. trichopterus* show developmental plasticity of the gills and labyrinth organ in response to chronic hypoxia. Several key questions arise from this study. Do the changes that occur in the gills and labyrinth in response to hypoxia actually enhance oxygen uptake from the environment? Are the observed responses proportional to hypoxia level, even to the extent that hyperoxia might actually suppress respiratory development? Finally, are these changes permanent, *i.e.* do they persist into adulthood even after return to normoxia, or are they maintained only under the pressure of chronic hypoxia? These and other questions deserve additional experimentation in the *T. trichopterus* and other air-breathing fishes.

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