

Developmental cardiorespiratory physiology of the air-breathing tropical gar, *Atractosteus tropicus*

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Burggren WW, Bautista GM, Coop SC, Couturier GM, Delgado SP, García RM, González CA. Developmental cardiorespiratory physiology of the air-breathing tropical gar, *Atractosteus tropicus*. *Am J Physiol Regul Integr Comp Physiol* 311: R689–R701, 2016. First published July 27, 2016; doi:10.1152/ajpregu.00022.2016.—The physiological transition to aerial breathing in larval air-breathing fishes is poorly understood. We investigated gill ventilation frequency (f_G), heart rate (f_H), and air breathing frequency (f_{AB}) as a function of development, activity, hypoxia, and temperature in embryos/larvae from day (D) 2.5 to D30 posthatch of the tropical gar, *Atractosteus tropicus*, an obligate air breather. Gill ventilation at 28°C began at approximately D2, peaking at ~75 beats/min on D5, before declining to ~55 beats/min at D30. Heart beat began ~36–48 h postfertilization and ~1 day before hatching. f_H peaked between D3 and D10 at ~140 beats/min, remaining at this level through D30. Air breathing started very early at D2.5 to D3.5 at 1–2 breaths/h, increasing to ~30 breaths/h at D15 and D30. Forced activity at all stages resulted in a rapid but brief increase in both f_G and f_H , (but not f_{AB}), indicating that even in these early larval stages, reflex control existed over both ventilation and circulation prior to its increasing importance in older fishes. Acute progressive hypoxia increased f_G in D2.5–D10 larvae, but decreased f_G in older larvae (\geq D15), possibly to prevent branchial O₂ loss into surrounding water. Temperature sensitivity of f_G and f_H measured at 20°C, 25°C, 28°C and 38°C was largely independent of development, with a Q₁₀ between 20°C and 38°C of ~2.4 and ~1.5 for f_G and f_H , respectively. The rapid onset of air breathing, coupled with both respiratory and cardiovascular reflexes as early as D2.5, indicates that larval *A. tropicus* develops “in the fast lane.”

development; heart rate; larval fish; respiration; tropical gar

AIR BREATHING HAS EVOLVED independently ~70 times in bony fishes (Osteichthyes) (11, 29). Within the lobe-finned fishes (Sarcopterygii), air breathing has evolved in three genera of lungfishes. Dipnoi possess true, paired lungs (except for *Neoceratodus* with a single lung) that are homologous with the lungs of higher vertebrates. Within the ray-finned fishes (Actinopterygii), air breathing is found within the reedfishes and bichirs (Polypteriformes within the Chondrostei), and within the Neopterygii, where air breathing occurs in numerous orders of Teleostei but also within the gars and bowfins (Holostei). As varied as the taxonomy of air-breathing in fishes are the organs they employ for aerial respiration, which can involve modification of the swim bladder, the gut (stomach and/or intestines),

the opercular chambers or structures within them, or true lungs, in the case of the lungfish.

The morphology and physiology of the terminal adult stages of numerous air-breathing fishes has been extensively investigated—for an introduction into that literature, the reader is directed to some key reviews (12–14, 29, 38–40, 49, 52, 64, 69, 70, 76–78, 84). Yet, despite considerable investigation of air-breathing fish, there are large gaps in our knowledge of these respiratory specialists. Consequently, Lefevre et al. (50) have recently indicated an urgent need for further information on the physiology, especially respiratory physiology, of air-breathing fishes, particularly those air breathers increasingly employed in aquaculture.

Despite the need for additional studies, the physiology of the embryos and larvae of air-breathing fishes has been only superficially investigated and is poorly understood (just as for the larvae of strictly aquatic fishes). The larvae are often the most vulnerable of the life stages, and natural selection operates heavily at this level (e.g., 41). Especially interesting but scarcely understood is the developmental transition in individual larvae from water breathing to combined water and air breathing and how the morphology, physiology, and reflex control of joint aquatic and aerial respiration come together in the process of respiratory maturation (31). To this point, however, most studies of the larval air-breathing fishes have focused on morphological descriptions of development, sometimes in the face of environmental stressors (e.g., temperature, oxygen level, pollutants) or nutritional variation, e.g., long nosed gar, *Lepisosteus osseus* (20, 53); Cuban gar, *Atractosteus tristoechus* (17); alligator gar, *Atractosteus spatula* (16, 21, 61); blue gourami, *Trichopodus trichopterus* (7, 8); Siamese fighting fish, *Betta splendens* (60); the arapaima, *Arapaima gigas* (28, 75); and various genera of lungfish (35, 43, 65, 90).

Even fewer studies have investigated the biochemistry and cellular and molecular biology of air-breathing larval fishes during their development, e.g., the snakehead *Channa punctatus* (2); the African catfish *Clarias gariepinus* (93); the tropical gar *Atractosteus tropicus* (3, 25, 30); the Florida gar *Lepisosteus platyrhincus* (26); alligator gar *Atractosteus spatula* (80); the Australian lungfish, *Neoceratodus forsteri* (91); the arapaima *Arapaima gigas* (10, 28), and the walking catfish *Clarias batrachus* (45).

Most infrequent of all in the larvae of air-breathing fishes are physiological studies at the organ system and organismal level. Metabolic rates, gill ventilation and heart rates, patterns of cutaneous blood flow, and the onset of air breathing and related parameters have been determined in a few species, such as the

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Australian lungfish, *Neoceratodus forsteri*; the blue gourami, *Trichopodus trichogaster*; the Siamese fighting fish, *Betta splendens*; and the swamp eel, *Monopterus* (8, 51, 59, 60, 65, 66). Yet, even basic elements of physiological development, such as the interrelationship between ontogeny and allometry for physiological rates, when and how physiological regulation of the respiration and circulation appear, and how physiological variables are affected by acute and chronic environmental stressors are lacking for larval air-breathing fishes.

Against this paucity of physiological information in larval air-breathing fishes, we have investigated aspects of the respiratory and cardiac physiology of the larvae of the air-breathing tropical gar *Atractosteus tropicus*, an obligate air breather as an adult. The Lepisosteiformes, comprising two genera and seven species ofgars, represents a particularly interesting group of air-breathing fishes with importance for recreational fishing, food consumption, and even culture (the prehistoric art of many Mesoamerican cultures depict the “pejelagarto” or tropical gar). *Atractosteus tropicus* has an extant distribution in eastern North America, Central America, the Caribbean Islands, with fossil gars recorded from Europe, the Asian subcontinent, and South America.

Various aspects of the physiology of juvenile and adult gars have been investigated (9, 15, 19, 27, 32, 48, 57, 73, 74, 79, 81, 82, 87, 88, 94–96). As typical for fishes generally, little is known of the physiology of the embryos and larvae of *A. tropicus*. More than just being one of many possible interesting air-breathing fishes in which to study physiological development, the tropical gar *Atractosteus tropicus* represents a fish with very rapid embryonic and larval development. This species in its natural habitat in Southern Mexico and Central America develops at temperatures in the range of 26–30°C or even higher, and accordingly the developmental progression through embryonic and larval stages is very rapid simply based on anticipated Q_{10} s for metabolism and development. Beyond “mere” rapid growth, however, larval *A. tropicus* begin to take air breaths as early as 4.5 days after fertilization, just 2.5 days after hatching and well before the yolk sac is absorbed and feeding has begun (Burggren W and Martínez G, unpublished data)! This compares with the onset of air breathing at about 32–44 days after hatching at similar temperatures in the air-breathing labyrinth fishes *Betta splendens* and *Trichopodus trichogaster* (60), 12 days in the air-breathing Thai pangas, *Pangasius sutchi* (36) and 9 days in *Arapaima gigas* (54). Graham (29) suggests that ~10 mm is the lower limit of size for the onset of aerial respiration in larval air-breathing fishes, and while *A. tropicus* is at this length or longer when it starts to air breathe, this gar does so within a few days of hatching! The larvae of the tropical gar, thus, presents the unusual opportunity to investigate the physiological transition from strictly aquatic gas exchange with skin and gills to mixed aquatic and aerial gas exchange with a gas bladder over a compressed period of chronological time in an air-breathing fish. As such, this species comprises a highly tractable model for studying the early physiological development of air breathing in a fish—a poorly understood process.

Importantly, the extremely rapid onset of air breathing in larval *Atractosteus tropicus*—assuming that air breathing is not solely for gas bladder inflation—suggests not only that the gas bladder has developed, but also that at least rudimentary vascularization of the bladder is present. A functional aerial

gas exchange organ in the form of the swim bladder with no associated cardio-respiratory reflexes would render aerial gas exchange at these early stages much less effective. Consequently, we thus hypothesized that larval *A. tropicus* would show reflex regulation of the both branchial and aerial ventilation at an equally early developmental stage. To test this hypothesis, gill ventilation rate and heart rate as a function of acute changes in temperature, oxygen levels, and activity levels (as well as air bladder ventilation following activity) have been measured in larval *A. tropicus* from embryonic stages through day 30 after hatching.

MATERIALS AND METHODS

Fish Rearing

Eggs and larvae were obtained from the aquaculture facility of La Universidad Juárez Autónoma de Tabasco in Villahermosa, Tabasco State, Mexico. All stages were simultaneously available from the aquaculture facility. Once yolk sac absorption occurred (approximately day 5), larvae were fed with brine shrimp (*Artemia* sp.), water fleas (*Daphnia* sp.), and powdered food, as described previously (56).

All stocks were maintained on an ~12:12-h light-dark cycle in nonchlorinated water at 27–28°C unless another temperature is indicated.

Histological material was prepared and photographed in 2006. Physiological experiments were carried out in November 2015. All experimental protocols for the physiology experiments were approved by the Research Administration of Laboratorio de Acuicultura de la Universidad Juárez Autónoma de Tabasco, Villahermosa, Mexico.

Length-Body Mass Measurements

To measure embryo length and mass (including yolk sac), embryos were removed from the chorion. Length of embryos, newly hatched and older larvae were measured from snout to tail end, with a digital caliper to the nearest millimeter. After carefully blotting to remove excess water, body mass was determined to the nearest milligram with an analytical balance. Sex could not be determined up to the stages examined in this study, so the data represent a mix of males and females.

Rate Measurements

Rates of heart beat, gill ventilation, and air breathing were determined as functions of development, temperature, activity, and oxygen level. Because an abundance of larvae and juveniles were available at all times, no fish was used for more than one type of measurement.

Gill ventilation. Gill ventilation frequency, f_G , was determined visually from the movements of the opercula. Larvae (excluding those from day 30) were placed in aerated, nonchlorinated water (except for hypoxia experiments) in size-appropriate plastic chambers (1 or 2 cm diameter, both sizes 1-cm depth) and observed under a dissecting microscope at $\times 5$ magnification. Day 30 fish were placed in 6-cm diameter clear polystyrene cups filled with ~2 cm of water (water volume ~100–125 ml), and their gill ventilation could be observed under normal room lighting conditions without necessity of a microscope. To maintain designated oxygen level and/or temperature, 25% of the water in the cups was very gently replaced via a syringe every 10 min during the measurement session. Care was taken to ensure that the water in the observation chamber was changed to the appropriate temperature and/or oxygen level without major disturbance of the fish, as evident by the lack of stimulation of locomotor activity or, at most, a short-lived (<4 s) locomotor response. Rates for ventilation (and for the other measured variables described below) are, thus, considered to be primarily “resting” rates. However, short periods of spontaneous swimming occasionally occurred during the observation periods, suggesting that the rates are more appropriately considered as “rou-

tine" rates. However, any fish that showed excessive or sustained swimming activity was eliminated from the experiment.

Air breathing. Air breathing frequency, f_{AB} , was determined from fish that were videoed while in the same observation chambers used for measurement of gill ventilation and heart rates. The videos were then visually analyzed for the release of air bubbles from the opercular chambers signifying a completed air breath. Air breathing frequency was calculated from all observed air breaths occurring during 15-min periods (see *Experimental Protocols* below).

Heart beat. Heart beat frequency, f_H , was determined visually under the microscope from the movements of the heart through the body wall. Larvae were placed in the same observation chambers used for measurement of gill ventilation, but they were placed over an angled mirror positioned to reflect the ventral surface of the fish into the microscope lens. Indirect lighting was usually most effective in highlighting heart movements, and was provided by the microscopes' built-in illumination. Heart rate was measured over two successive 15-s periods, with the two values averaged to generate the average heart rate for a given fish under a given condition at a given time.

Experimental Protocols

Enforced activity. Gill ventilation rates in normoxia at 28°C were recorded when fish were first placed in the observation chambers and then after 15, 30, and 45 consecutive minutes. Fish were then mechanically disturbed with a blunt probe for 1 min, essentially mimicking a "startle" response. They were then immediately assessed for gill ventilation frequency and then again at 15, 30, and 45 consecutive minutes for a total of 8 measurements covering preactivity and postactivity.

Measurement of air breathing rates before, immediately after, and following recovery followed a similar protocol as for gill ventilation, except that air breathing was recorded at the end of consecutive 15-min periods rather than instantaneously as for f_G .

Heart rates were recorded 5 min after placement in a recording chamber, immediately after enforced activity induced, as described above, and then 2–4 min following activity.

Acute temperature change. Fish in the observation chambers were cooled through the gentle introduction of cold, normoxic water from a syringe until water temperature reached 20°C, at which point f_G and f_H were determined. The water was then warmed by gentle addition of water in 5-min intervals to 25°C, 28°C, and 38°C, with a measurement of f_G and f_H as each temperature was reached, before returning the fish to 28°C. This process typically resulted in little or no locomotor activity in the fishes in their individual containers, especially upon cooling. Water temperatures were measured with an electronic thermometer (YSI model 55 digital oxymeter; Yellow Springs Instruments, Yellow Springs, OH).

Temperature sensitivity of gill ventilation and heart beat rates was expressed as the rates Q_{10} , calculated thusly:

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{\left(\frac{10}{T_2 - T_1} \right)} \quad (1)$$

Acute oxygen change. Fish in the observation chambers were measured for f_G in normoxic water at 28°C, over two successive 15-min periods. The PO_2 of the water in the observation chambers was then lowered in a step-wise fashion to 10.6 kPa, after which rates were measured six times at 5-min intervals. PO_2 level was then further lowered to 2.5 kPa, followed by an additional six rate measurements at 5-min intervals. A final rate measurement was made at 1.2 kPa before PO_2 was returned to normoxic levels.

The effects of hypoxia on f_H were assessed by making a measurement of routine f_H and then lowering PO_2 to 3.1 kPa, followed after 5 min by a second measurement at this level of hypoxia.

Histology

Larvae initially preserved in 4% formalin buffered to pH 7.6 were dehydrated with graded series of ethanol concentrations and then embedded in paraffin with an automatic tissue processor (ZX-60, Histolab, Göteborg, Sweden). Paraffin blocks were then prepared in AP280-2 station and cut into 3- μ m-thick serial sagittal or transverse sections with an automatic microtome (Microm HM, Thermo Scientific). Paraffin cuts were kept overnight at 40°C. Samples were then deparaffined with a graded series of xylene concentrations and stained by a standard haematoxylin and eosin (H&E) used for general histomorphological observations. Histological preparations were observed in a Leica DMLB microscope equipped with an Olympus DP70 digital camera (Leica Microsystems, Nussloch, Germany).

Statistics

Assessment of the significance of the effects on f_G , f_{AB} , or f_H of activity, temperature, or hypoxia at the measured developmental stages was made with repeated-measures two-way ANOVA, followed by Holm-Sidak test for multiple comparisons. A significance level of 0.05 was used for all tests. Analyses were conducted using SigmaPlot Version 12.3 software.

RESULTS

Length-Body Mass Relationships

Length and wet body mass relationships for *A. tropicus* from hatching to 30 days old are shown in Fig. 1. As length increased, so too did body mass increase in a predictable pattern, following a quadratic relationship. However, while there was a very high correlation coefficient for this relationship, the relationship between chronological age and body mass was far more ambiguous. Above 30–40 mm in length, individual fish from the same age class could exhibit a twofold difference in wet body mass, reflecting differences in feeding and food assimilation and, especially, the propensity toward cannibalism, which led to large differential rates of body mass increase among this group.

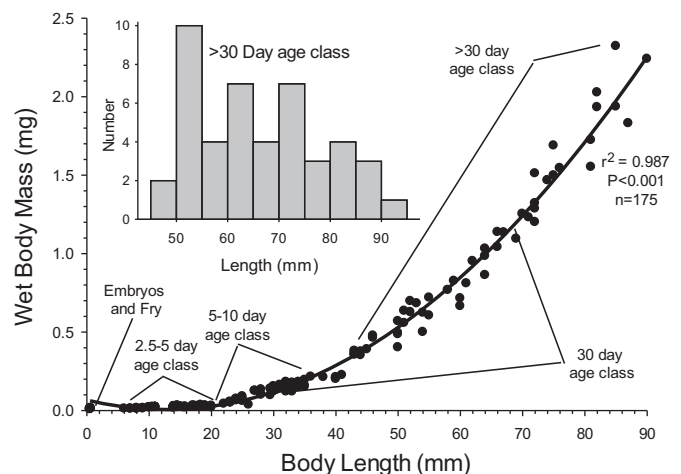


Fig. 1. Length-body mass relationship in embryonic, larval, and early juvenile *A. tropicus* reared at 28°C. Embryos were removed from the chorion, but otherwise were weighed with intact yolk sac. The fitted line represents a quadratic equation. The 95% confidence intervals are too small to be visualized. *Inset*: frequency histogram in the >30-day age class. Sex could not be determined at these developmental stages, so data represent a mix of males and females.

Ventilation Rates

Gill ventilation. Developmental changes in routine f_G are shown in Fig. 2A. Gill ventilation commenced at *day 2.5* (D2.5 henceforth) of posthatch development. Initially, opercular

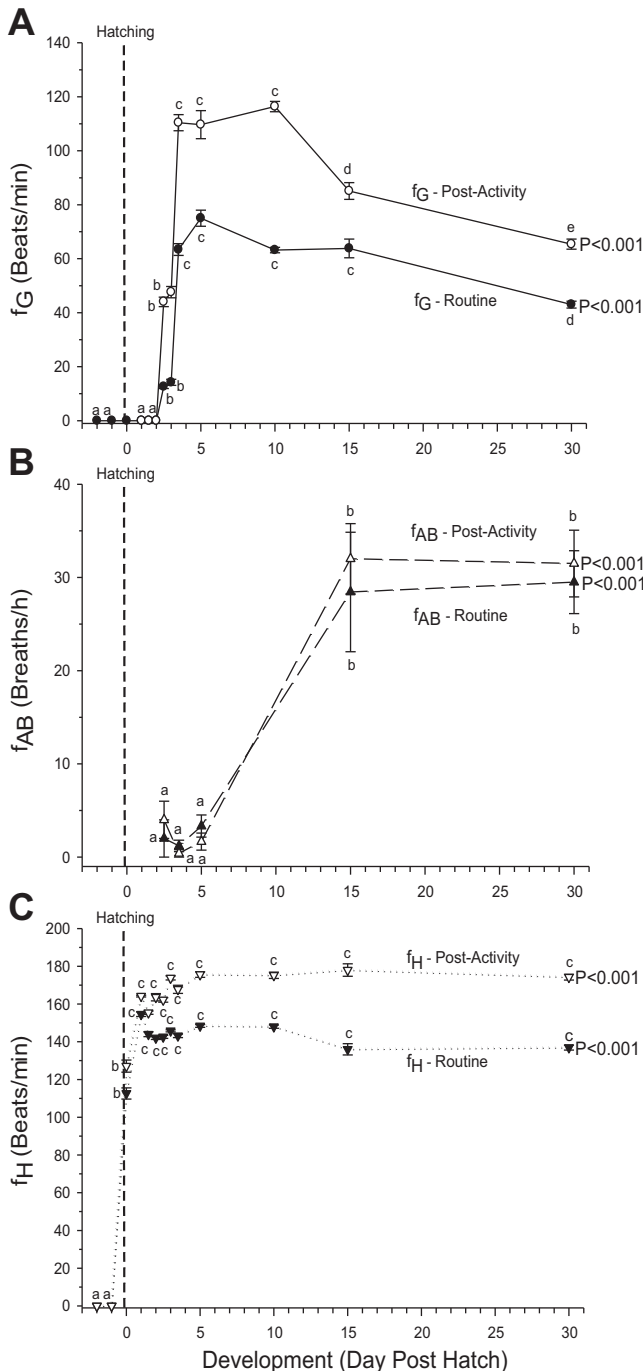


Fig. 2. Frequencies of gill ventilation frequency, f_G (A), air breathing, f_{AB} (B), and heart rate, f_H (C), at during routine conditions of limited, spontaneous activity (Routine) and after 1 min of enforced activity (Post-Activity) in *Atractosteus tropicus* at designated developmental stages. Measurements were made in normoxia at 28°C. Mean values \pm SE are presented. $n = 8$ for D4, D15, and D30. $n = 10$ for all other stages. The P value to the right of each plot indicates the significance level for the effect of development on the indicated variables. Different letters within a developmental stage signify significant differences at least a $P < 0.05$ level.

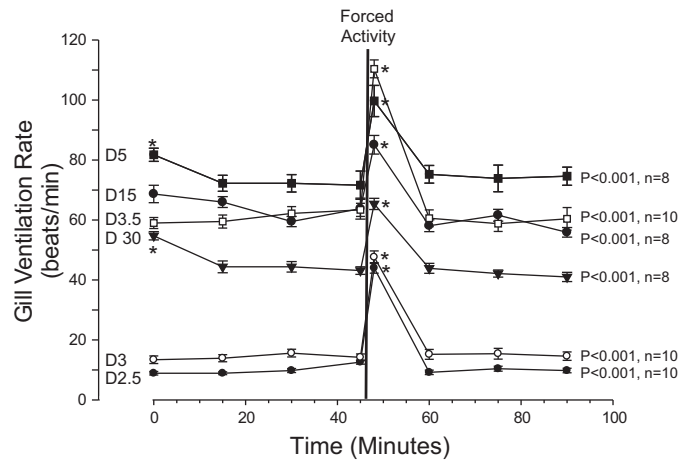


Fig. 3. Time course of the effects on gill ventilation frequency of 1 min of enforced activity in *day 2.5* (D2.5) to *day 30* (D30) *Atractosteus tropicus* in normoxia at 28°C. Mean values \pm SE are presented. Significance values to the right of each plot indicate for the overall effect of activity across time at each developmental stage. *Significant ($P < 0.001$) difference from the routine f_G value at time = 45 min, immediately prior to induced activity. Forced activity significantly elevated f_G in all developmental stages. See text for additional details and statistical analyses.

movements were irregular, but within a few hours of the initiation of gill ventilation, opercular movements became more regular, albeit at a low frequency of 1–15 beats/min at D2.5 to D3. Ventilation in resting fish subsequently rose quickly to a peak of 75–80 beats/min at D5. From D5 through D30 routine f_G declined to ~ 50 beats/min.

Enforced activity significantly ($P < 0.001$) elevated f_G by ~ 20 – 25% in all stages examined, including D2.5 when gill ventilation first occurred (Fig. 2). Indeed, the proportional increase in f_G was largest in the youngest animals. The specific time course of the effects of 1 min of enforced activity are shown in Fig. 3. Upon placement in the measurement chamber (likely resulting in some disturbance), f_G was initially significantly elevated in *days 3.5, 5, and 30*. However, in all days of development, f_G stabilized over the first hour of measurement, such that values at time = 45 min (designated the “routine” value) were generally among the lowest rates recorded. One minute of enforced activity resulted in an immediate and significant ($P < 0.001$) increase in f_G in all measured developmental stages (Fig. 3). However, the stimulation of f_G was short-lived, as values of f_G in all stages returning to preactivity, routine levels within 15 min of the cessation of activity.

Not surprisingly, routine f_G was sensitive to temperature at all developmental stages (Fig. 4), with a significant interaction ($P < 0.01$) between rate and day of development. Generally, the highest Q_{10} values for f_G were found in the youngest larvae at the lowest temperatures, with values of ~ 6.5 to 9, but these values diminished at the highest temperature interval. This pattern of greatest temperature sensitivity for f_G tended to repeat for older larvae (Table 1). Q_{10} values for routine f_G over the total temperature range of 20 to 38°C ranged from 2 to 3 (Fig. 5).

Hypoxia significantly affected gill ventilation at all stages, but the effects were qualitatively, as well as quantitatively different depending upon developmental stage. Two distinct patterns of ventilatory response to progressive hypoxia were observed. In the earlier developmental stages, routine f_G in-

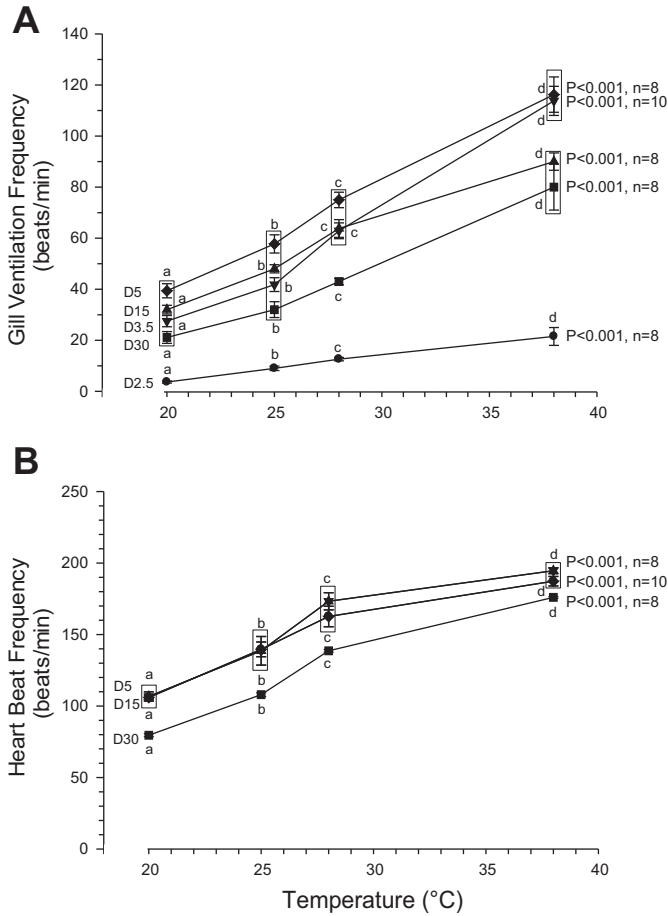


Fig. 4. Effects of acute temperature change on routine gill ventilation frequency (A) and heart rate (B) in day 2.5 (D2.5) to day 30 (D30) in *Atractosteus tropicus*. Mean values \pm SE are presented. Significance values for the overall effect of temperature at each developmental stage are indicated. Different letters within a developmental stage signify significant differences at $P < 0.05$. Boxes enclose means within each temperature that are not significantly different. See text for additional details.

creased significantly ($P < 0.01$) in response to hypoxia (Fig. 6A). For D2.5 larvae, a significant increase in f_G representing nearly a tripling of rate occurred at 2.5 kPa, but at the most severe hypoxia level (1.2 kPa) f_G declined sharply back to normoxic levels. In D3.5, D5, and D10 larvae, each downward step in oxygen level stimulated an overall significant stepwise increase in f_G . However, the response to progressive hypoxia was qualitatively different in fish from D15 and D30. Upon first encountering mild hypoxia, f_G increased as in the earlier stages, but in D15 fish, f_G returned to normoxic levels as hypoxia became more severe (Fig. 6B). In D30 fish, the greater hypoxic levels actually reduced f_G to levels at or below values in recorded in normoxia.

Gas bladder ventilation (air breathing). Air breathing was observed in a few larvae as young as D2.5, although regular air breathing at a low level of 2–4 breaths/h typically began between D3.5 and D5 (Fig. 2B). By D15, routine air breathing frequency increased significantly ($P > 0.05$) to ~20–25 breaths/h, remaining at this level at D30.

Unlike gill ventilation frequency, air breathing frequency was not significantly ($P > 0.05$) affected by enforced activity at any level of development (Fig. 7).

Table 1. Q_{10} for gill ventilation and heart frequency in *Atractosteus tropicus* in normoxia

Stage	Q_{10} for Gill Ventilation Rate				Q_{10} for Heart Rate			
	20–25°C	25–28°C	28–38°C	20–38°C	20–25°C	25–28°C	28–38°C	20–38°C
D2.5	8.87 \pm 2.47**† (n = 8)	6.47 \pm 3.55 (n = 10)	1.58 \pm 0.15 (n = 8)	2.92 \pm 0.28 (n = 8)	1.74 \pm 10 (n = 6)	1.67 \pm 0.09 (n = 6)	1.16 \pm 0.04 (n = 6)	1.37 \pm 0.01 (n = 6)
D3.5	2.77 \pm 0.60 (n = 10)	6.47 \pm 3.55 (n = 10)	1.72 \pm 0.11 (n = 10)	2.26 \pm 0.13 (n = 10)	1.74 \pm 10 (n = 6)	1.67 \pm 0.22 (n = 6)	1.16 \pm 0.04 (n = 6)	1.37 \pm 0.01 (n = 6)
D5	2.50 \pm 0.61 (n = 8)	2.92 \pm 0.93 (n = 8)	1.52 \pm 0.11 (n = 8)	1.89 \pm 0.10 (n = 8)	1.74 \pm 0.24 (n = 6)	1.67 \pm 0.22 (n = 6)	1.16 \pm 0.09 (n = 6)	1.40 \pm 0.01 (n = 6)
D15	2.48 \pm 0.38 (n = 8)	2.84 \pm 0.58 (n = 8)	2.44 \pm 0.09 (n = 8)	2.67 \pm 0.10 (n = 8)	1.86 \pm 0.09 (n = 9)	2.32 \pm 0.09 (n = 9)	1.22 \pm .01 (n = 9)	1.56 \pm .0 (n = 9)
D30	5.00 \pm . (n = 8)	2.98 \pm 0. (n = 8)	1.50 \pm 0.12 (n = 8)	2.67 \pm 0.10 (n = 8)	NS	NS	NS	NS
Significance for each temperature	$P > .05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	NS	NS	NS	NS

Values are expressed as means \pm SE. NS, not significant. *Significant difference compared with Day 5. †Significant difference compared with 25–28°C within stage.

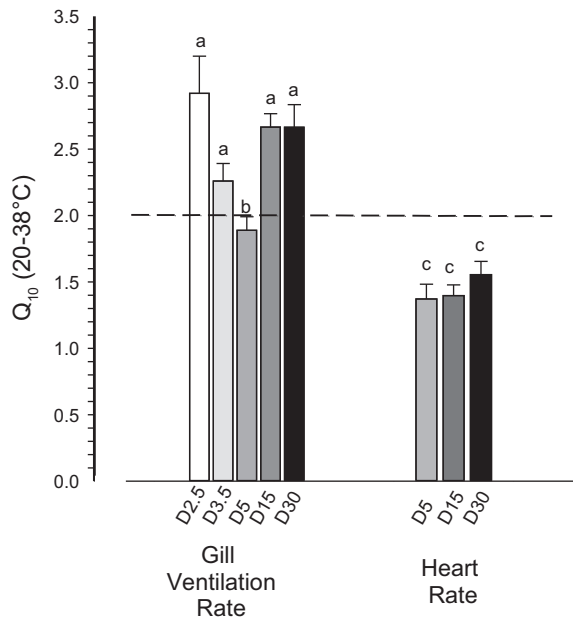


Fig. 5. Q_{10} for routine gill ventilation frequency and heart rate in day 2.5 (D2.5) to day 30 (D30) *Atractosteus tropicus* in normoxia. Q_{10} s are indicated for the overall temperature range of 20°C to 38°C. Mean values \pm SE are presented. Different letters signify significant differences at $P < 0.05$. For gill ventilation, $n = 8$ for each developmental stage except for D3.5, where $n = 10$. For heart rate, $n = 10$ for all stages. Refer to Table 1 for Q_{10} s for smaller temperature intervals between 20°C and 38°C.

Heart Beat

Routine heart rate of 110–130 beats/min was first detectable around the point of hatching (D0), and rose significantly ($P < 0.01$) to 140–150 beats/min by D2, a rate that was then maintained through D30 (Fig. 8).

Just as for gill ventilation, f_H was significantly increased ($P < 0.01$) by 1 min of enforced activity at all measured developmental stages, increasing by ~ 20 –25% at all measured stages (Fig. 2C; Ref. 9).

Temperature increased heart rate as expected, with f_H rising significantly from ~ 100 beats/min at 20°C to ~ 175 beats/min at 38°C (Fig. 4B). Q_{10} values for f_H over the large temperature range of 20 to 38°C were ~ 1.5 at each of the three developmental stages for which Q_{10} was measured (Table 1, Fig. 5). Noteworthy is that Q_{10} values for f_H were significantly lower than the Q_{10} values for f_G over the range 20°C to 38°C.

Hypoxia (3.1 kPa) resulted in either small or nonsignificant increases in f_H in early stages, with larger increases at D5 through D30 (Fig. 9). Notably, however, even in those stages with significant increases in f_H induced by hypoxia (D2.5, D3.5, D4, D5, D10, and D15), the increases were much smaller than the increases in f_H produced by enforced activity.

Larval Cardiorespiratory Morphology

Aspects of embryonic and larval morphology are indicated in Fig. 9. Eggs are translucent to transparent (Fig. 9, A and B). While pale upon hatching (Fig. 9C), larvae quickly develop pigmentation (Fig. 9, D and E). Not only is the yolk sac heavily vascularized (Fig. 9E), but so too are several areas of the cutaneous surfaces, especially the thin, flattened tail (Fig. 9F) and the dorsal surface of the head (Fig. 9G). The

opercula develop within a few days of hatching, but still only partially enclose and cover the gill arches by D3 (Fig. 9, G and H). By D13 to D15, the characteristic long snout has developed (Fig. 9I).

The gills are quite rudimentary through the first days of development, with little apparent surface area (Fig. 10, A and B). By D9, the gills have begun elongation, and gill filaments have begun bearing lamellae (Fig. 10C). The gas bladder is well formed and large as early as day 4 (Fig. 10D). Swellings in the gas bladder wall appear to be muscular bundles (arrows), potentially apparent on D4 (Fig. 10B) and more evident by D14 (Fig. 10, E and F). By D14, the gas bladder is lobed caudally (Fig. 10F).

DISCUSSION

Implications of Developmental Changes in Body Mass, Length, and Shape

Growth morphometrics have been investigated as a function of normal development, as well as modified temperature and diet in several gar species, including the alligator gar, *Atractosteus spatula* (1, 62), the Cuban gar, *Atractosteus tristoechus* (17, 18), and the tropical gar, *Atractosteus tropicus* (1, 25). Body mass changes during development for *A. tristoechus* (17, 18) and *Atractosteus tropicus* (1, 25) show somewhat similar patterns to the closely related *A. tropicus* presented in the current study. However, an increase in body mass began about D2 after hatching in the Cuban gar, suggesting that some limited feeding must be occurring prior to yolk sac absorption in that species. In the tropical gar, although body length was steadily increasing in the first days after hatching, body mass was not (25) (Fig. 1), suggesting that mass was simply being transferred from yolk material into actual body tissue in the absence of feeding. An overall increase in mass did not begin to occur until complete yolk sac absorption had occurred by about D5.

Relationships between day of development and either body mass or body length are difficult to interpret, at least in *Atractosteus tropicus*, simply because the same age class of larvae can have very large variations in body mass and length (Fig. 1). These variations may be due to different rates of success in making the transition to feeding, as well as the tendency toward cannibalism, which leads to especially rapid growth (56). Thus, the relationship portrayed in Fig. 1 likely conveys a more accurate sense of growth than graphs of mass vs. age or body length vs. age employed in other studies on this species (25). A future study looking at rates of assimilation and growth in larval tropical gar as a function of feeding, temperature, and oxygen levels would be very instructive.

Larval *A. tropicus* have a highly elongate, fusiform body shape. Despite the thickly scaled skin of adults, the early larvae have highly vascularized skin (Fig. 9, E–G). Combined, these characteristics likely allow the body surface of larval gar to provide for considerable gas exchange. Interestingly, in D2 to D3 larvae, the rapidly beating pectoral fins generated a flow of water posteriorly along the body surface. This is reminiscent of the flow of water generated along the body surface of the larvae of the air-breathing swamp eel *Monopterus* (51) which, in conjunction with posterior to anterior blood flow, generated a counter blood flow and water flow that is highly efficient for cutaneous gas exchange. A study in larval *A. tropicus* that

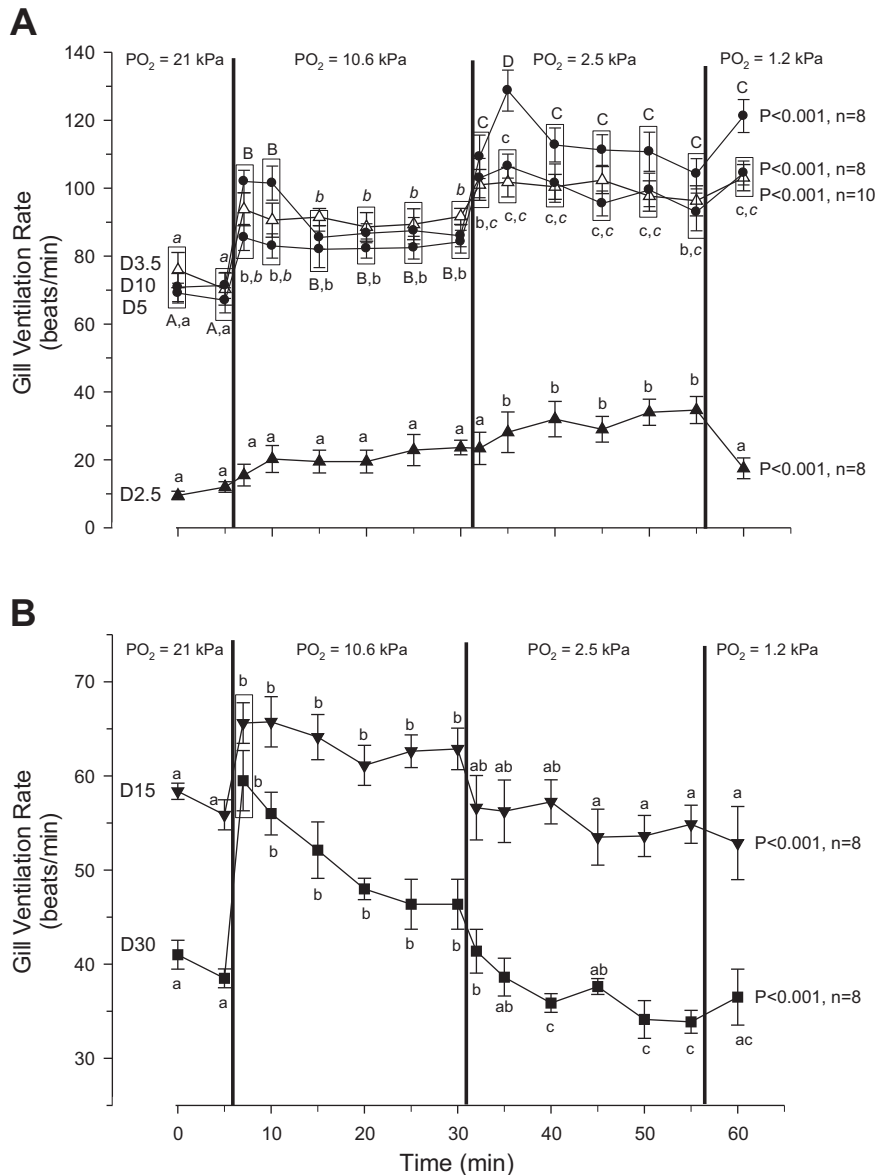


Fig. 6. Routine gill ventilation frequency related to progressive, acute hypoxic exposure in *Atractosteus tropicus* at 28°C. **A:** day 2.5, 3, 5 and 10, where progressive hypoxia stimulated f_G . **B:** days 15 and 35, where progressive hypoxia caused an initial rise in f_G followed by a decline to levels at or below those in normoxia. Mean values \pm SE are presented. Different letters signify significant differences at $P < 0.05$; in A, D3.5, D5 and D10 are depicted with lower case, lower case italic, and upper case letters, respectively.

partitions gas exchange between branchial, cutaneous, and aerial gas exchange surfaces would identify the role of cutaneous vs. branchial and air bladder gas exchange.

Gill and Gas Bladder Morphology

The gills of the larvae of tropical gar are rather rudimentary up until at least D9. Gill filaments I–IV are present as early as D2 (Fig. 10A), but by D9, the gill filaments are still relatively short and the lamellae are sparse and widely separated along the filaments (Fig. 9H and 10C). Many air-breathing fishes, both facultative and obligative air breathers, possess reduced gills structures—and, thus, reduced gill surface area—as both larvae and adults (14, 29, 38, 77). Such reduction of gill surface area helps to reduce the specter of oxygen loss from oxygenated blood returning from the gas bladder across the gills and out into hypoxic water. Reduced branchial surface area, coupled with the reduced branchial ventilation rates in hypoxia (see below), may be quite effective in optimizing overall gas exchange in hypoxic environmental conditions.

As anticipated from the relatively early onset of air breathing behavior as early as D2.5, the bladder is inflated with air along its length by D4 (Fig. 10D). Transverse thin sections through the body of D14 larvae reveal a well-developed gas bladder (Fig. 10, E–G) that is lobed caudally (G). Interestingly, muscle-like structures appeared in the bladder walls by at least D14 (arrows in Fig. 10, E and F), although the presence of muscle was not investigated histologically. However, smooth muscle elements have been described in the gas bladder walls of adult *L. oculatus* (34). The presence of smooth muscle (in addition to the smooth muscle of the vasculature), suggests that gar may be able to compress or relax the bladder. Such movements potentially would change the volume of the gas bladder, which could, in turn, be used as a mechanism for adjusting buoyancy while submerged. Additionally (or alternatively), regional contraction and relaxation of the gas bladder could affect some stirring/mixing of gas within the lobed gas bladder, which might aid gas exchange during submergence.

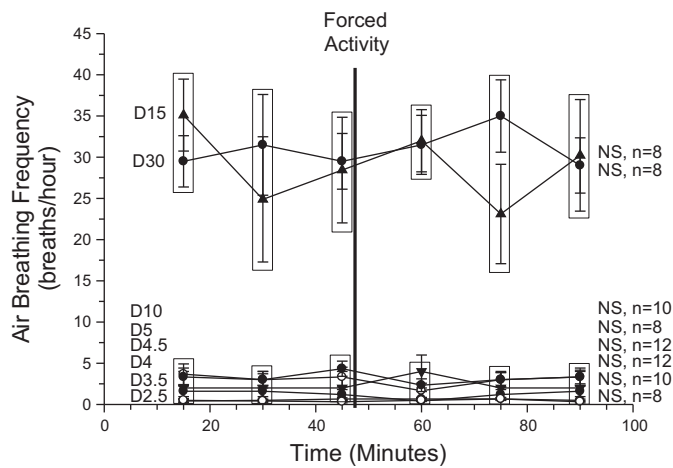


Fig. 7. Time course of the effect on routine air breathing frequency of 1 min of forced activity in day 2.5 (D2.5) to day 30 (D30) *Atractosteus tropicus* in normoxia at 28°C. Mean values \pm SE are presented. Boxes enclose means at each measurement time that are not significantly different. Although f_{AB} was significantly higher in D15 and D30, forced activity had no significant effect ($P > 0.05$) on routine rates at any stage of development. Significance and n values on the right are stacked in the same order as the symbol keys on the left.

Temperature and Physiological Rates

Not surprisingly, increases in both gill ventilation and heart rate were positively correlated with ambient temperature (Figs. 4, 5, and 8), but the greatest temperature sensitivity was evident at the lower temperature intervals. Interestingly, heart rate increased to a lesser extent than gill ventilation, as temperature increased (Fig. 5, Table 1). The increase in heart rate between 28°C and 38°C was lower than between 20°C and 25°C or 25°C and 28°C (Table 1, Fig. 4B). This plateauing at higher temperatures may reflect an absolute upper limit on the rate at which the heart can beat and maintain normal filling and ejection functions. Indeed, heart rates in adult fishes (excepting tuna) are typically below 120 beats/min (22, 85). f_H in larval fishes generally peak at \sim 200 beats/min at temperatures above 30°C (4), and this level may represent the maximum effective heart rate for a fish heart of this size and structure. Noteworthy is that a similar plateauing of heart rate with increasing temperature, albeit at much lower temperatures and cardiac frequencies in the range of 60–70 beats/min, occurs in the brook char, *Salvelinus fontinalis*, as temperature increases (6).

Ventilation-perfusion matching is an important component of effective gas exchange, and it is interesting to note that increases in heart rate “fall behind” the increases in gill ventilation as body temperature rises in *A. tropicus*. However, rates alone do not necessarily directly reflect the volume of branchial irrigation and perfusion, as there is a complex, species-specific relationship between heart rate, stroke volume, and cardiac output in fishes (as described in Refs. 24, 47, and 86).

Enforced Activity and Physiological Rates

Gill ventilation rate was stimulated by enforced activity (a startle), either resulting from initial placement in the observation apparatus (D5, D30) or from actual enforced activity following 45 min without disturbance (all days of development) (Fig. 3). This response was short-lived, however, and at

all days of development, f_G returned to routine, preactivity values within 15 min of activity. These findings indicate that at least a preliminary reflex control of branchial ventilation exists, even at the earliest stages, just hours after gill ventilation has begun. The onset of air breathing quickly follows the onset of gill ventilation (Fig. 2), so the presence of at least partially developed branchial reflexes would help optimize gas exchange involving both gills and air bladder, especially when larvae are in severely hypoxic water (see below).

Routine rates of air breathing in larval tropical gar increased as development proceeded, but there was no significant effect of enforced activity on air breathing frequency at any developmental stage (Figs. 2B and 8). Given that activity in both normoxic and hypoxic water increases air breathing frequency in adult gars—at least the spotted gar (15)—it is interesting to speculate on this lack of air breathing response when gill ventilation and heart rate clearly are stimulated by activity in larval *A. tropicus*. One explanation stems from the fact that gars place a priority on acid-base balance vs. oxygen uptake in the face of short-term environmental modifications (15). Aerial hyperventilation during and immediately after activity in larval *A. tropicus* could contribute to hypocapnia—especially if accompanied by branchial hyperventilation—which, in turn, would raise blood pH. Thus, the increase in aerial oxygen uptake would occur at the cost of a disturbance to acid-base balance in these larvae. Another explanation might be that in the earliest stages of development, there is no respiratory role for the bladder (perhaps air breathing is for bladder inflation and buoyancy), or the reflexes controlling air breathing develop later than those controlling gill ventilation. Finally, an alternative (or additional) explanation may be found not in cardiorespiratory physiology, but rather in predator avoidance behavior. Surfacing for air breathing exposes especially small air-breathing fish to the risk of aerial predation from birds and other predators (92). Since gill ventilation (and presumably branchial gas exchange) increases briefly with enforced activity, the lack of stimulation of air breathing in small, vulnerable

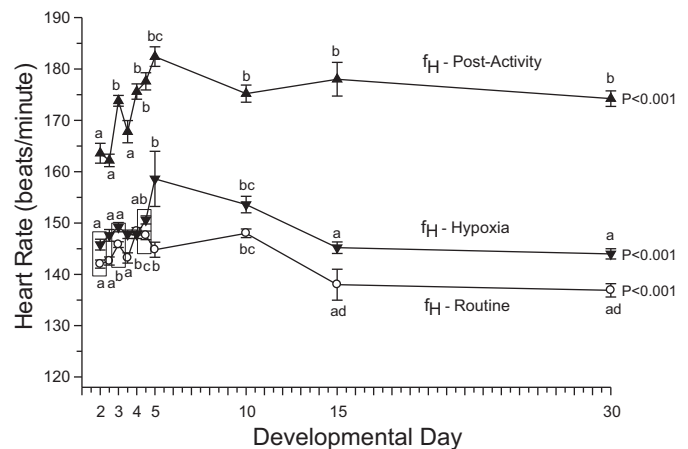


Fig. 8. Effects of acute hypoxia (\sim 3 kPa) on routine heart beat frequency as a function of development in *Atractosteus tropicus* in normoxia at 28°C. Mean values \pm SE are presented. Different letters within a developmental stage signify significant differences at $P < 0.05$. Boxes enclose means within each developmental stage that are not significantly different. $n = 10$ for each developmental stage. Heart rate changes induced by acute enforced activity, already illustrated in Fig. 2C, are replotted for reference. See text for additional details.

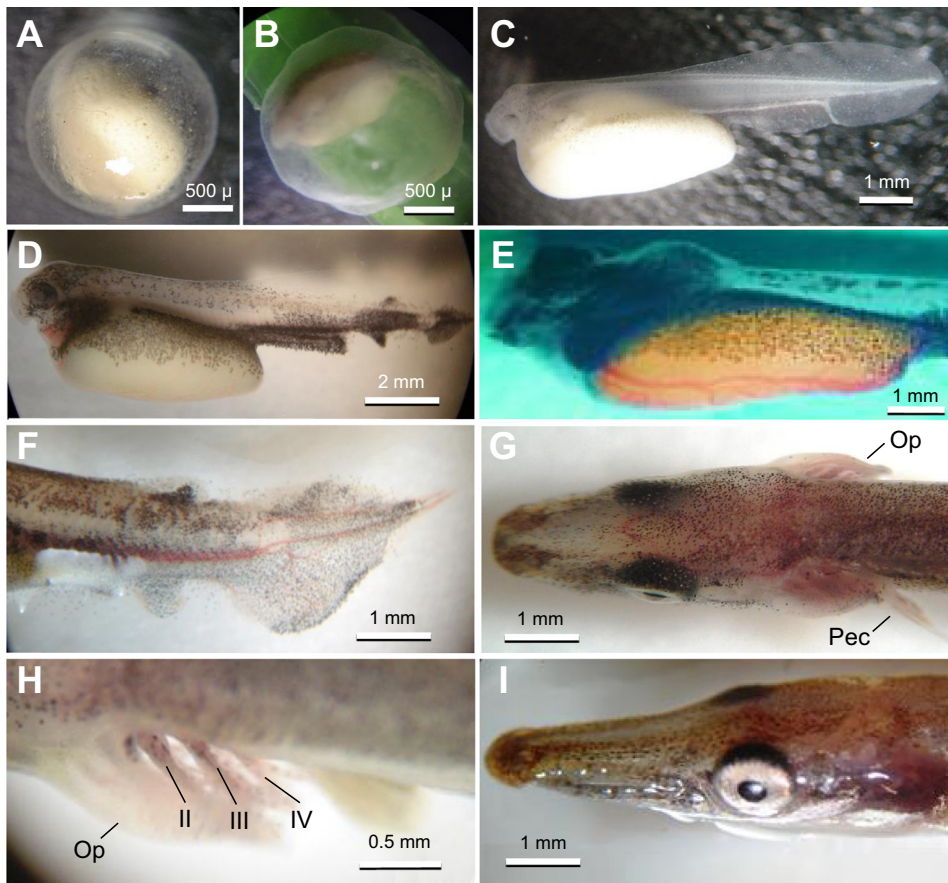


Fig. 9. Embryonic (A and B), newly hatched (day 0) (C), day 2 posthatch (D and E), day 3 (F–H), and days 13–15 (I) tropical gar, *Atractosteus tropicus*. Op, operculum; Pec, pectoral fin. II, III and IV indicate gill arch numbers.

larval stages could be an adaptation to reduce (or at least not increase) the risk of aerial predation. In adult spotted gar, ventilation of the air bladder is stimulated by exhaustive exercise (15) or by the sudden switch from dark to light associated with an experimental photoperiod (81). Presumably, the tropical gar develops reflex control over air bladder ventilation relatively early in its development, but additional experiments will be required to reveal the developmental onset of such regulation.

Enforced brief activity causes a large, significant increase in heart rate, in the range of a 20–30% increase (Fig. 2C). These effects were larger than those induced by acute, severe hypoxia at all developmental stages (Fig. 8). Interestingly, the cardiovascular effects (heart rate and dorsal aortic blood pressure) of exercise in adult *Lepisosteus oculatus* were also relatively minor (15). A cardiac “startle response” has also been documented in larval zebrafish, where it is attributed to the presence of both sympathetic and parasympathetic branches of the autonomic nervous system (55).

Acute Hypoxia and Physiological Rates

Gill ventilation frequency was significantly affected by acute, progressive hypoxia, but the qualitative nature of the responses was highly stage-specific (Fig. 6). From the onset of gill ventilation at D2.5 through D10, hypoxia generally caused an increase in f_G , with more severe hypoxia causing more pronounced increases. The severest hypoxia level (~ 1 kPa) only failed to stimulate f_G at a level of 1.2 kPa in the youngest larvae examined, where the decrease in f_G may have actually

represented a ventilatory depression in larvae too young to overcome this severe hypoxia. Such ventilatory depression at relatively extreme oxygen levels has also been recorded in the larvae of strictly aquatic teleosts such as the rainbow trout (33). Hypoxic ventilatory stimulation has been documented in the larvae of numerous aquatic freshwater teleosts, beginning as early as 2 days postfertilization and more fully developed by 7–9 dpf in the zebrafish (*Danio rerio*) (42, 71, 72, 83), which has been most extensively studied in this regard.

In the older larvae (D15 and D30), however, progressive hypoxia caused an initial increase in f_G that was then followed by a decline—in the case of D30 to levels that were at or below routine f_G levels in normoxia. All larvae of these later stages recovered upon return to normoxia, so it is unlikely that this was a direct depression of gill ventilation so much as a reflexly triggered reduction. What might be the purpose of such a reduction in gill ventilation in severe hypoxia? Air-breathing fish of all stripes face the specter that oxygen gained through aerial respiration can be lost through aquatic ventilation when in severely hypoxic water. In almost all species, oxygenated blood from the aerial gas exchange organ arrives via systemic venous drainage into the heart and is then immediately propelled onto the gills, where it could be lost with a “reverse” flow of oxygen down a P_{O_2} gradient from branchial afferent blood across the branchial exchange surfaces to the severely hypoxic water irrigating the gills (14, 89). In the gars, the entire cardiac output must flow through the gills, there being no specialized shunt vessels to provide a branchial bypass. However, in many air-breathing fishes, the posterior gill arches have

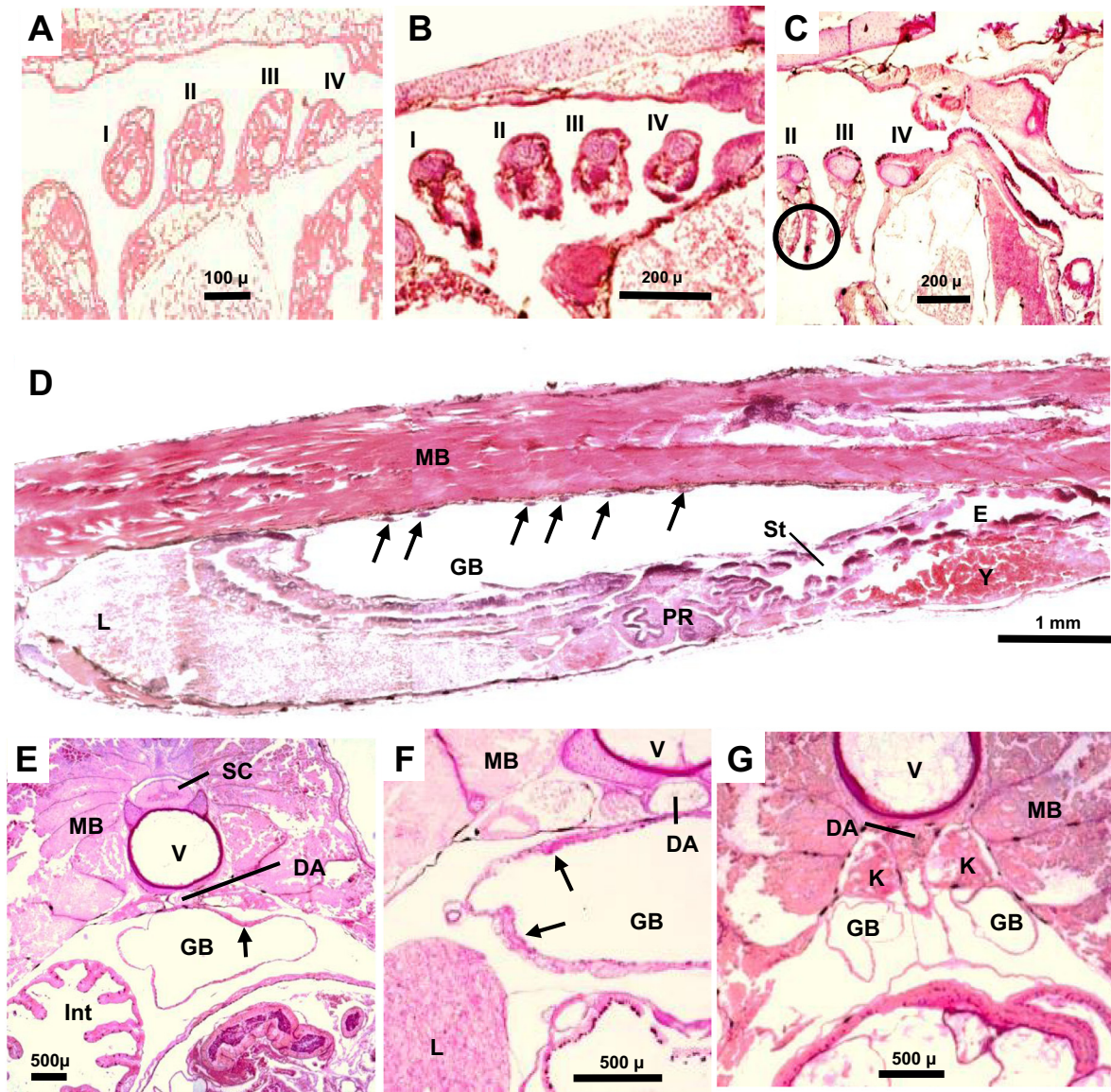


Fig. 10. Sagittal and transverse sections through the body of the tropical gar, *Atractosteus tropicus*. Sagittal thin sections through the branchial chamber are shown for day 2 (A), day 3 (B), and day 9 (C). D: sagittal thin section through the body of a day 4 larva. Transverse thin sections through the body of day 14 larvae (E and F), with G representing a more caudal plane than E and F. Arrows indicate possible developing structure within the walls of the gas bladder. CD, central duct of gas bladder; DA, dorsal aorta; E, esophagus; GB, gas bladder; Mb, muscle bundles; L, liver; Op, operculum; Pec, pectoral fin; PR, pyloric region of the stomach; S, thick septa of gas bladder; SC, spinal cord; St, stomach; V, vertebral column. Arrows indicate possible muscular structures within the gas bladder walls. I, II, III, IV indicate gill arches. The circle in C encloses filaments with visible lamellae.

a reduced surface area to some extent, and oxygenated venous blood from the heart can be preferentially shunted through these rear arches to mitigate oxygen loss to the surrounding water (29, 38, 77). Smatresk and Cameron (89) suggest that the vascular arrangement in the spotted gar does not enable such shunting. We have not examined branchial morphology of *A. tropicus* in detail and, thus, cannot comment on the (likely) absence of such shunts in this species. There is, however, rather reduced branchial structure in larvae (Fig. 10, A–C; see discussion above), which would reduce branchial surface area. Even in the absence of branchial surface area reduction, there is little doubt that the decrease in f_G seen in D15 and D30 *A. tropicus* will likely reduce overall branchial irrigation, unless there is some offsetting increase in branchial stroke volume.

Such reduction in branchial irrigation will potentially limit loss of oxygen across the gills into the surrounding hypoxic water.

Heart rate showed significant increases with hypoxia at most but not all developmental stages, through D30 (Fig. 8). However, the induced tachycardia was small at most stages, and certainly smaller than that induced by enforced activity. In the rainbow trout, an hypoxic tachycardia occurs during the first 8 days of development (33), although this switches to a bradycardic response with further development (63). Indeed, a hypoxic bradycardia is typical of many adult fishes exposed to hypoxia, including strictly aquatic fishes (23, 44, 68) and most bimodal breathers (5, 67). The heart rate of larval zebrafish exposed to hypoxia during the first 2 wk postfertilization has been reported to either show a tachycardia (37) or remain

unchanged (4), and then at later stages, a hypoxia bradycardia develops (46). Few data on cardiac responses to hypoxia are available for gars of any developmental stage, but it is noteworthy that aquatic hypoxia had little or no effect on heart rate in the adult longnose gar (89).

Development and Maturation of the Cardiorespiratory Regulation

Both branchial ventilation and heart beat respond to enforced activity and to hypoxia with rate increases in larvae of the tropical gar, *Atractosteus tropicus*. These cardiorespiratory responses to both activity and aquatic hypoxia strongly indicate the existence of physiological reflexes, which, in turn, indicates the existence of both sensory receptors that sense the internal and external environment, as well as the motor elements of the reflexes that modulate the key physiological processes of ventilation and circulation. The absence of anything but developmentally related changes in air ventilation rates does not negate this conclusion, but rather points to the complex interaction between aquatic and aerial gas exchange, as well as possible behavioral components (e.g., avoidance of predation). The appearance of these reflexes within hours of hatching, along with the rapid growth and maturation of the larvae, supports the conclusion that the tropical gar is highly precocious relative to many other fishes. As such, it will be interesting to investigate other aspects of its physiology in a comparative framework. Finally, of interest is the considerable variation in air-breathing frequencies (Figs. 2 and 7) compared with the other measured physiological rates. Certainly, some larvae were breathing steadily by D3, while others were still taking only infrequent breaths at this stage. This may simply reflect intraspecific differences in the onset of the neuromuscular control and mechanism for air breathing. Recently, intraspecific differences in “boldness” involved in air breathing have been shown in the catfish, *Clarias gariepinus* (58). Perhaps such “personality” differences affecting behavior and physiological rates arise quite early in the tropical gar, as well.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

W.W.B., G.M.B., S.C.C., G.M.C., S.P.D., R.M.G., and C.A.A.G. conception and design of research; W.W.B., G.M.B., and S.C.C. performed experiments; W.W.B., G.M.B., and S.C.C. analyzed data; W.W.B., G.M.B., S.C.C., and C.A.A.G. interpreted results of experiments; W.W.B. prepared figures; W.W.B. drafted manuscript; W.W.B., G.M.B., S.C.C., G.M.C., S.P.D., and C.A.A.G. edited and revised manuscript; W.W.B., G.M.B., S.C.C., G.M.C., S.P.D., and C.A.A.G. approved final version of manuscript.

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