CARDIO-RESPIRATORY ONTOGENY AND THE TRANSITION TO BIMODAL RESPIRATION IN AN AIR BREATHING FISH, THE BLUE GOURAMI (*Trichogaster trichopterus*): MORPHOLOGICAL AND PHYSIOLOGICAL DEVELOPMENT IN NORMOXIA AND HYPOXIA

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As selection pressures exist for not only adults, but for every life history stage, it is important to understand how environmental factors shape developing animals. Despite the significance placed on aquatic hypoxia as a driving force in the evolution of air breathing, this is the first known study to examine the effects of hypoxia on cardio-respiratory ontogeny of an air breathing fish.

Blue gouramis are obligatory air breathing fish that possess a labyrinth-like structure that serves as the air breathing organ. Gouramis were reared for up to 90 d in normoxia or hypoxia, and morphological and physiological development was observed. Hypoxic larvae had increased lamellar and labyrinth organ surface areas. Bradycardia and increased gill ventilation rates were observed when larvae from either rearing group were briefly exposed to hypoxia. Hypoxic larvae also showed a reduced heart rate and gill ventilation rate in the absence of a hypoxic stimulus, possibly indicative of a more comprehensive, long-term respiratory plasticity. The similarity of routine oxygen consumption between rearing groups suggests that metabolic demand did not change for hypoxic larvae, but that they were more efficient at oxygen acquisition. This is further supported by increased resistance time of hypoxic gouramis to extreme hypoxia. The onset of air breathing was between 20 and 25 d post-fertilization, and was not affected by either rearing or exposure environment. It may be that this behavior is associated
with the inability of smaller larvae to successfully overcome water surface tension, rather than with the necessity of aerial respiration at this stage.

Hypoxia is commonly experienced by most air breathing fishes, and studies of hypoxia-induced developmental effects may provide critical insights into the evolution of air breathing. The studies presented here provide novel data on the plasticity of cardio-respiratory development of an air breathing fish reared in hypoxia, and can serve as a solid foundation for future studies.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>CHAPTERS</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. MORPHOLOGICAL DEVELOPMENT OF THE RESPIRATORY STRUCTURES OF THE BLUE GOURAMI</td>
<td>13</td>
</tr>
<tr>
<td>3. PHYSIOLOGICAL DEVELOPMENT OF THE CARDIO-RESPIRATORY SYSTEMS OF THE BLUE GOURAMI</td>
<td>56</td>
</tr>
<tr>
<td>4. HYPOXIA-INDUCED DEVELOPMENTAL PLASTICITY OF RESPIRATORY STRUCTURES OF THE BLUE GOURAMI</td>
<td>82</td>
</tr>
<tr>
<td>5. HYPOXIA-INDUCED DEVELOPMENTAL PLASTICITY OF CARDIO-RESPIRATORY PHYSIOLOGY OF THE BLUE GOURAMI</td>
<td>119</td>
</tr>
<tr>
<td>6. IMPLICATIONS OF RESEARCH AND FUTURE DIRECTIONS</td>
<td>148</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>155</td>
</tr>
</tbody>
</table>
LIST OF TABLES

2.1 Abbreviations of morphological parameters studied...........................35

2.2. Summary statistics of whole body measurements of blue gouramis reared in normoxia through 90 dpf.................................................................36

2.3. Summary statistics for gill measurements of gouramis reared in normoxia through 90 dpf...............................................................................................37

2.4. Summary statistics for labyrinth surface area and total respiratory surface area of gouramis reared in normoxia through 90 dpf.......................................38

3.1. Abbreviations of physiological parameters studied..............................75

4.1. Summary statistics for whole body measurements of gouramis reared in normoxia or hypoxia through 90 dpf.................................................................99

4.2. Summary statistics for gill measurements of gouramis reared in normoxia or hypoxia through 90 dpf..............................................................................100

4.3. Summary statistics for additional gill measurements of gouramis reared in normoxia or hypoxia through 90 dpf.................................................................102

4.4. Summary statistics for labyrinth surface area and total respiratory surface area of gouramis reared in normoxia or hypoxia through 90 dpf..................104

5.1. Summary statistics for cardio-respiratory physiological development........138
LIST OF FIGURES

1.1. Relative position and structure of gills and labyrinth organ of Anabantids...........12

2.1. Photographs of gill arches III-VI of a juvenile blue gourami.........................39

2.2. Techniques of measurements of the gills of developing blue gourami..............40

2.3. Branchial dissections of juvenile blue gouramis of the same brood showing growth of the labyrinth organ in relation to body size........................................41

2.4. Total body length (A) and total body wet mass (B) as function of development of gouramis reared in normoxia through 90 d post-fertilization (dpf)......................42

2.5. Total body length as function of total body wet mass of gouramis reared in normoxia through 90 dpf..........................................................43

2.6. Cutaneous surface area as function of development (A) and total body wet mass (B) of gouramis reared in normoxia through 90 dpf.....................................44

2.7. Gill arch length (A) and filament length (B) as function of total body wet mass of gouramis reared in normoxia through 90 dpf............................................45

2.8. Number of filaments by gill arch (A) and total number of filaments per animal (B) as function of total body wet mass of gouramis reared in normoxia through 90 dpf..........................................................46

2.9. Filament density by gill arch as function of total body wet mass of gouramis reared in normoxia through 90 dpf..........................................................47

2.10. Lamellar count by gill arch (A) and total body lamellar count (B) as function of total body wet mass of gouramis reared in normoxia through 90 dpf.............48

2.11. Lamellar density by gill arch as function of total body wet mass of gouramis reared in normoxia through 90 dpf..........................................................49

2.12. Single lamellar surface area by gill arch (A) and total lamellar surface area by gill arch (B) as function of total body wet mass of gouramis reared in normoxia through 90 dpf..........................................................50
2.13. Total body lamellar surface area as function of total body wet mass for gouramis reared in normoxia through 90 dpf..........................................................51

2.14. Total labyrinth surface area as function of total body wet mass of gouramis reared in normoxia through 90 dpf..........................................................52

2.15. Respiratory surface area by (A) structure and (B) total respiratory surface area as function of total body wet mass of gouramis reared in normoxia through 90 dpf........................................................................53

2.16. Mass-specific respiratory surface area by structure as function of total body wet mass of gouramis reared in normoxia through 90 dpf........................................................................54

2.17. Extrapolations of mass-specific relative respiratory surface area by structure as function of total body wet mass of gouramis reared in normoxia through 90 dpf........................................................................55

3.1. Heart rate as a function of development of blue gourami larvae reared in normoxia through 15 dpf..................................................................................76

3.2. Gill ventilation rate as function of development of blue gourami larvae reared in normoxia through 15 dpf..................................................................................77

3.3. Onset of air breathing as stimulated by extreme hypoxia (aquatic and aerial) as function of development of gouramis reared in normoxia through 60 dpf..............78

3.4. Air breathing frequency measured in normoxia (aquatic and aerial) as function of development of blue gouramis reared in normoxia through 60 dpf..............79

3.5. Total oxygen consumption as function of (A) development and (B) total body wet mass of blue gouramis reared in normoxia through 60 dpf..............................80

3.6. Mass-specific oxygen consumption as function of (A) development and (B) total body wet mass of gourami reared in normoxia through 60 dpf..............................81

4.1 Morphological development of gourami larvae reared from <24 h post-fertilization (hpf) through 132 hpf in two levels of hypoxia (10% O₂ and 13% O₂) and normoxia (21% O₂)..................................................................................................................105

4.2. Total body length (A) and total body wet mass (B) as function of development of gouramis reared in hypoxia through 90 dpf.........................................................106

4.3. Total body length as function of total body wet mass of gouramis reared in hypoxia through 90 dpf.........................................................................................107
4.4. Cutaneous surface area as function of (A) development and (B) total body wet mass of gouramis reared in hypoxia through 90 dpf..............................108

4.5. Gill arch length (A) and filament length (B) as function of total body wet mass of gouramis reared in hypoxia through 90 dpf..............................109

4.6. Number of filaments by gill arch (A) and total number of filaments per animal (B) as function of total body wet mass of gouramis reared in hypoxia through 90 dpf.................................................................110

4.7. Filament density by gill arch as function of total body wet mass of gouramis reared in hypoxia through 90 dpf.................................................................111

4.8. Lamellar count by gill arch (A) and total body lamellar count (B) as function of total body wet mass for gouramis reared in hypoxia through 90 dpf................112

4.9. Lamellar density by gill arch as function of total body wet mass of gouramis reared in hypoxia through 90 dpf.................................................................113

4.10. Single lamellar surface area (A) and total lamellar surface area (B) by gill arch as function of total body wet mass of gouramis reared in hypoxia through 90 dpf..................................................................................114

4.11. Total body lamellar surface area as function of total body wet mass of gouramis reared in hypoxia through 90 dpf.................................................................115

4.12. Total labyrinth surface area as function of total body wet mass of gouramis reared in hypoxia through 90 dpf.................................................................116

4.13. (A) Total respiratory surface area as function of total body wet mass of gouramis reared in hypoxia through 90 dpf. (B) Percent contribution to total respiratory surface area by each reaspiratory structure for both rearing groups............117

4.14. (A) Mass-specific surface area of respiratory structures and (B) Total respiratory surface area as function of total body wet mass of gouramis reared in hypoxia through 90 dpf.................................................................118

5.1. Heart rate measured in normoxia (21% O₂) as function of development of gourami larvae reared in hypoxia through 15 dpf..............................................139

5.2. (A) Heart rate measured in moderate hypoxia (13% O₂) as function of development of gourami reared in normoxia or hypoxia through 15 dpf. (B) Heart rates of both rearing groups exposed to either normoxia or moderate hypoxia.................................................................140
5.3. Gill ventilation rate measured in normoxia as function of development of gourami reared in hypoxia through 15 dpf…………………………………………………………..141

5.4. (A) Gill ventilation rates measured in moderate hypoxia as function of development of gouramis reared in normoxia or hypoxia through 15 dpf. (B) Gill ventilation rates for both rearing groups exposed to either normoxia or moderate hypoxia………………………………………………………………………………..142

5.5. Air breathing frequency measured in normoxia (aquatic and aerial) as function of development of gouramis reared in hypoxia through 60 dpf……………………………………143

5.6. (A) Air breathing frequency measured in extreme hypoxia (~3-5% O_2; aquatic and aerial) as function of development of gouramis reared in normoxia or hypoxia through 60 dpf; (B) Air breathing frequency for both rearing groups exposed to either normoxia or extreme hypoxia………………………………………………………………………………..144

5.7. Time to loss of equilibrium in extreme hypoxia (aquatic and aerial) as function of development of gouramis reared in normoxia or hypoxia through 60 dpf……..145

5.8. Total oxygen consumption as function of (A) development and (B) total body wet mass for gouramis reared in hypoxia through 60 dpf……………………………………146

5.9. Mass-specific oxygen consumption as function of (A) development and (B) total body wet mass of gouramis reared in hypoxia through 60 dpf……………………………..147
1.1. The Evolution of Air Breathing

Animals that respire solely in water face different challenges than those able to breathe air. Water has a higher viscosity and density than air, and thus also has a greater resistance to flow through respiratory pathways (Johansen, 1970; Dejours, 1994; Nikinmaa, 2002). These properties require that an aquatically respiring animal routinely expend more metabolic energy, not only as it propels itself through the water, but also as it moves water over its respiratory surfaces (Holeton, 1980; Schmidt-Nielsen, 1997). Additionally, water contains less dissolved oxygen than air per unit volume, due to the solubility coefficient of oxygen (Johansen, 1970; Dejours, 1994; Nikinmaa, 2002).

Air breathing fish, while maintaining some level of metabolism via aquatic respiration, are also capable of taking advantage of atmospheric air. Thus they can acquire a greater amount of oxygen at a lower cost than completely aquatic breathers (Randall et al., 1981; Kramer, 1983; Schmidt-Nielsen, 1997). The ability to breathe air requires specialized organs or systems that are highly vascularized with small air-blood diffusion barriers to ensure proper gas exchange, yet are resilient enough to maintain their structure, despite the influences of low density and net gravitational forces of air (Johansen, 1970; Banerjee, 2007; Ong et al., 2007).

Air breathing in vertebrates is believed to have first appeared in fishes of the Late Silurian Period about 400 million years ago, and has since occurred independently at
least 49 times along different lineages in the Class Osteichthyes, resulting in 370 known species of air breathing fishes (Graham, 1997). Most air breathing freshwater fish are found in tropical regions, where they routinely experience hypoxia induced by high temperatures, seasonal drought, and high biological oxygen demand (Dehadrai and Tripathi, 1976; Graham, 1997; Chapman et al., 2002). The lower layers of a typical tropical swamp are often completely deoxygenated (Rai and Munshi, 1979; Munshi and Hughes, 1992). It is generally agreed that air breathing in vertebrates arose as a result of aquatic hypoxia (Carter, 1957; Johansen, 1970; Randall et al., 1981; Graham, 1997).

Even in hypoxic waters, the uppermost layer of water is usually well oxygenated, and many water breathing animals take advantage of the air-water interface by employing aquatic surface respiration (Kramer and McClure, 1982; Gee and Gee, 1995; Florindo et al., 2006). Fishes performing aquatic surface respiration invariably take in some air, which is usually immediately released from the mouth or operculum (Peters, 1978). In the goldfish (Carassius auratus), for example, “air gulping” results in increased arterial-venous oxygen saturation in extreme hypoxia, thus improving blood oxygen transport during hypoxic exposure (Burggren, 1982). Such benefits of surface respiration may have been the impetus for the evolution of specialized chambers or organs to utilize the inhaled air for respiration (Gee and Gee, 1995; Graham, 1997), and thus may have been the first step on the evolutionary path toward air breathing.

The lengthy and diverse evolution of air breathing has resulted in various methods for taking advantage of atmospheric air. This diversity is well represented by the lifestyle differences observed in these fishes. Air breathing fishes can, at the most basic level, be divided into amphibious air breathers and aquatic air breathers. Twenty-
four families of fishes contain “amphibious air breathers”, defined as fish that are able to survive via aerial respiration when out of water. Some examples include the mudskipper (*Periophthalmus*) and the lungfishes (*Lepidosiren* and *Protopterus*). Aerial exposure may be forced, as with seasonal drought, or may be a behavioral action, such as excursions out of water to search for food or a less crowded environment. Aquatic air breathing fishes are those that remain submerged in water but come to the surface to gulp air. This category can further be divided into facultative air breathers, which only breathe air under stressful conditions such as decreased environmental oxygen or increased oxygen requirements, and continuous air breathers, which gulp atmospheric air continuously (although not always at a constant frequency) despite oxygen levels or requirements. Continuous air breathers can be either obligatory, meaning that they would be incapable of survival in normoxic water if denied access to atmospheric air, or non-obligatory, signifying that survival is possible in normoxic water even if atmospheric air is not accessible (see Graham, 1997)

Before discussing the blue gourami (*Trichogaster trichopterus*) as the air breathing fish of choice for this study, let us first consider the developmental biology of fishes of the suborder Anabantoidei, to which it belongs.

1.2. Anabantid Development

Anabantid fishes are paired spawners (Linke, 1991), with all aspects of spawning incited by the male, including oogenesis in the spawning females (Degani and Schreibman, 1993; Degani, 2001). A male establishes territory in a small pond or slow moving stream and builds a bubble nest. He then encourages a female to join him and
breeding commences (Degani, 1989, 2001; Linke, 1991). Breeding takes place in a temperature range of 23°C to 29°C (Degani, 1989). Following fertilization of the eggs, the male will usually chase away the female and will collect the embryos into the bubble nest, which he maintains and defends (Picciolo, 1964; Linke, 1991; Dzieweczynski et al., 2005). Replenishing the nest with fresh, oxygen-rich bubbles is crucial, as this presumably protects the embryos from the detrimental effects of hypoxia, common in the water below (Mol, 1993; Hostache and Mol, 1998). For example, in the armored catfish *Hoplosternum littorale*, another bubble nest builder, removal of the adult male results in the flattening of the nest, which had previously maintained a dissolved oxygen content of 6.5 to 7.5 mg O$_2$ l$^{-1}$. The eggs subsequently deteriorate, likely due to contact with the hypoxic water (0.5 to 3.0 mg O$_2$ · l$^{-1}$) (Hostache and Mol, 1998). Similar levels of hypoxia are found in breeding areas of the Anabantid *Betta splendens* (3.79±2.87 mg O$_2$ · l$^{-1}$) (Jaroensutasinee and Jaroensutasinee, 2001).

At about 1 d post-fertilization (dpf), the embryos hatch within the bubble nest, and will remain there for the first day or two post-hatch, until yolk reserves are depleted and the larvae must begin foraging for food (Hisaoka and Firlit, 1962). Embryos and young larvae are entirely aquatic and rely upon diffusion alone to meet their oxygen requirements. As body size increases, the cardiovascular system begins to contribute to oxygen distribution, and the later development of the gills leads to active oxygen uptake via branchial respiration (see Rombough, 2007). When the diffusion distances at the skin and gills increases to the point that oxygen uptake across these surfaces is no longer adequate, the developing suprabranchial chambers and labyrinth organs take over, and the transition to air breathing commences (Prasad, 1988; Graham, 1997). For
Anabantids, this transition usually takes place at 18-20 dpf, when the animal has reached a total body length of 10-12 mm (Das, 1927; Peters, 1978; Graham, 1997).

1.3. The Blue Gourami, *Trichogaster trichopterus*, as an Experimental Animal

*Trichogaster trichopterus* (Figure 1.1A) is of the suborder Anabantoidei, which consists of five families native to Southeast Asia (Das, 1927; Peters, 1978; Graham, 1997). Fish of this suborder are referred to as “labyrinthine” or “labyrinth” fishes, because the air breathing organ consists of a labyrinth-like structure located in paired suprabranchial chambers, residing behind the opercula and above the gills (Figure 1.1B). Adult *T. trichopterus* belongs to the category of continuous, obligatory air breathers. However, the developmental progression towards this lifestyle has not previously been determined, and thus larval and juvenile blue gourami, upon attaining the ability to breathe air, may more appropriately reside along a continuum, ranging from the immature (e.g. facultative) to adult strategy.

*Trichogaster trichopterus* is well suited for laboratory research for a number of reasons. Popular as an aquarium species, they are readily available at local pet stores. This species is quite hardy and quickly acclimates to most aquatic conditions. Blue gouramis can be bred year-round, and although embryos are delicate and early larval mortality rates are high, this is countered by the high fecundity of adult females (Hisaoka and Firlit, 1962; Degani and Schreibman, 1993; Degani, 2001). Additionally, larvae remain virtually transparent for up to two weeks, allowing for visual observations of internal morphology or physiological processes such as heart rate. Embryonic stages and basic aspects of early larval development have been described by Hisaoka and
Firlit (1962): When reared at 27°C, gourami larvae hatch approximately 24 h post-fertilization (hpf). Newly hatched larvae are buoyant and are maintained in the bubble nest, or float upside-down at the surface of the water until 36 hpf, when they right themselves and swim freely. The yolk is mostly consumed by 72 hpf, at which time larvae begin feeding.

1.4. Research Objectives

Air breathing in fishes likely arose as a result of frequent exposure to aquatic hypoxia (Carter, 1957; Johansen, 1970; Graham, 1997; Graham and Lee, 2004; Flück et al., 2007). As selection pressures exist for not only adults, but for every life history stage, it is important to understand how environmental factors shape developing animals. Despite the significance placed on aquatic hypoxia as a driving force in the evolution of air breathing, no known study has examined the developmental effects of hypoxia on respiratory ontogeny or the transition to bimodal respiration in any species of air breathing fish. Thus, I employed the blue gourami (*Trichogaster trichopterus*) as an experimental animal for studying morphological and physiological development of cardio-respiratory systems in response to chronic hypoxia. While some knowledge of the adult physiology exists for this species (e.g. Burggren, 1979; Burggren and Haswell, 1979; Herbert and Wells, 2001), virtually nothing is known of its morphological or physiological ontogeny. Therefore, I first detail cardio-respiratory development of the blue gourami reared in normoxia (Chapters 2, 3), and then move on to discuss the effects of chronic hypoxia on its morphological (Chapter 4) and physiological (Chapter 5) ontogeny, with special reference to the transition to bimodal respiration.
In the first study (Chapter 2 – Morphological Development of the Respiratory Structures of the Blue Gourami), I report morphological development of the whole animal and of the respiratory structures of blue gouramis reared under normoxic conditions. I hypothesized that total body length, wet mass, and cutaneous surface area would, of course, increase with development. As cutaneous respiration is the sole method of gas exchange in early larvae, the skin was expected to have the greatest mass-specific surface area (mm$^2$·g$^{-1}$ body mass), but was predicted to decrease as the animal grew larger and gills began to develop. I expected the branchial surface area to then increase quickly throughout larval development, with the gills growing steadily relative to the whole animal until reaching the juvenile stage (20-30 dpf), at which time aquatic respiration would become more limited due to the development of scales, reduced mass-specific surface area of the gills, and possibly a thickening of the blood-water barrier at the secondary lamellae. I predicted that at this point in development, the suprabranchial chambers and labyrinth organs are capable of functioning as respiratory organs, and thus air breathing would commence.

The second study (Chapter 3 – Physiological Development of the Cardio-Respiratory Systems of the Blue Gourami) includes details of the normoxic physiological ontogeny of the cardio-respiratory systems, including heart rate, gill ventilation rate, aquatic and aerial oxygen consumption rates, onset and frequency of air breathing, and hypoxia resistance. Heart rate and mass-specific oxygen consumption were expected to increase very early in development, around the time of hatching, and then to steadily decrease with increasing mass (Brown and West, 2000; Burggren, 2005). Gill ventilation was expected to begin at 3-5 dpf, concomitant with the development of the
primary and secondary lamellae, and that the rate of branchial respiration would increase steadily throughout early larval development. Air breathing was not expected to commence until around 20 dpf, at which time the frequency of aerial respiration would initially increase as the animal habituated to this lifestyle, followed by a steady decline to the rate reported for adults (Burggren, 1979). I hypothesized that resistance to extreme hypoxia (aquatic and aerial; defined as time to loss of equilibrium) would increase throughout early development, while the animal is still completely aquatic but as respiratory needs are increasing. As the animal becomes more dependent upon aerial respiration, however, I hypothesized that this resistance time would decrease.

In the third study (Chapter 4 – Hypoxia-induced Developmental Plasticity of Respiratory Structures of the Blue Gourami), I compare morphological development of blue gourami larvae and juveniles reared in hypoxia with data from those reared in normoxia (Chapter 2). I hypothesized that hypoxic exposure would speed development of the respiratory organs, such that lamellar and labyrinth surface areas would be augmented above those of larvae reared in normoxia.

In Chapter 5 (Hypoxia-Induced Developmental Plasticity of Cardio-Respiratory Physiology of the Blue Gourami), I compare physiological development of blue gourami larvae and juveniles reared in hypoxia with that of larvae raised in normoxia (Chapter 3). I hypothesized that the increased growth of respiratory surfaces observed in Chapter 4 would translate to an earlier onset of air breathing for larvae reared in hypoxia. I did not expect mass-specific oxygen consumption to differ between groups, only that the methods of acquiring this oxygen would be altered when acutely exposed to varying oxygen levels. For example, I predicted that when exposed to normoxia, hypoxia-
reared larvae would show decreased heart rate (bradycardia), gill ventilation rate and air breathing frequency. I predicted that the opposite would occur when normoxia-reared larvae were placed in acute hypoxia, i.e. increased heart rate (tachycardia), gill ventilation rate and air breathing frequency. I also expected that these parameters would be similar between groups when each rearing group was acutely exposed to its respective oxygen level. Additionally, I hypothesized that resistance to extreme hypoxia would be greater in the hypoxia-reared group, as evidenced by an increased time to loss of equilibrium.

The following section (Materials and Methods) details general procedures used in all experiments of the remaining Chapters (2-5).

1.5. Materials and Methods

1.5.1. Maintenance and Breeding of Adults

Adult blue gouramis (*Trichogaster trichopterus*) were purchased from local retailers and maintained in 38 L glass aquariums, with 3 to 5 adults per tank. Tank water was prepared by combining 2 g Instant Ocean (Aquarium Systems USA) with 1 L de-ionized water to achieve a 0.2% NaCl solution. This salt treatment was used to control external parasites and protect fish against stresses brought about by frequent handling (Swarm and Fitzgerald, 1992). Water was maintained at 27°C and tanks were refilled with de-ionized water as needed to maintain an adequate depth and salt concentration. Each tank was equipped with a Penguin 330 (Marineland) filter. Tanks were siphoned twice each week to remove excess food and waste, and up to 50% water changes were performed once per week, or as conditions necessitated
(determined by weekly water tests for pH and ammonia/nitrate/nitrite). Adults were routinely fed Tetramin dry fish flakes once daily. When being conditioned to breed, feedings increased to twice daily, and included Tetramin flakes, fresh and frozen brine shrimp, and frozen bloodworms.

After conditioning a pair of adults for one to two weeks, the male was placed in a separate 38 L glass aquarium (breeding tank) with many plants and a floating substrate, under which the male could build a bubble nest. In nature the male uses this nest to maintain and care for the offspring during the first few days of development (Degani, 1989, 2001). After allowing the male to become accustomed to his new surroundings, usually six to eight hours, the female was introduced into the breeding tank. Within a few hours to a few days the male built the nest and encouraged the female to begin breeding. Following successful breeding(s), both adults were removed from the breeding tank and replaced into their respective holding tanks.

1.5.2. Care and Maintenance of Larvae

Embryos were removed from the bubble nest within 12 h of fertilization and transferred to an 18 L glass tank filled with ~3 L tank water. The tank was then placed into a larger (38 L) sealed glass tank in order to maintain high humidity in the air above the water, which has been suggested as beneficial for larvae as they transition to air breathing (Degani, 2001; Alderton and Gibbs, 2008). Air was bubbled through the water of the larval tank to maintain normoxia (20.9% O₂). The tank was maintained in an incubator set at 27.0°C (Hisaoka and Firlit, 1962; Degani, 1991; Degani, 2001), and this temperature was monitored daily using 1°C-increment thermometers.
Larvae were fed a mixture of Tetramin dry fry food, Cyclop-Eeze, and Spirulina three times daily until approximately two weeks of age, at which time they were large enough to consume freshly hatched *Artemia* twice daily from then on. Water was siphoned and replaced twice each week, or as conditions necessitated (as determined by weekly tests of pH and ammonia/nitrite/nitrate levels). The water level of the larval tank was increased throughout development to avoid high densities that may have compromised larval growth.
Figure 1.1. (A) Adult blue gourami, *Trichogaster trichopterus*. (B) Relative position and structure of labyrinth organ of blue gourami, as observed from suprabranchial dissection of juvenile (60 dpf; 16.3 mm total body length). Also seen are the eye and first gill arch (GA-III).
CHAPTER 2
MORPHOLOGICAL DEVELOPMENT OF RESPIRATORY STRUCTURES
OF THE BLUE GOURAMI

2.1. Introduction

Air breathing fishes have long captured the imagination of comparative physiologists (e.g. Owen, 1841; Boake, 1865; Dobson, 1874; Zograff, 1888; Morris, 1892). Unfortunately, we know little other than some basic observations of the respiratory organs of most air breathing fish, and quantitative developmental data is almost entirely lacking. Yet, like the larvae of many other Teleost fishes, they are produced in great numbers and are transparent in early stages, facilitating their study. One group of interest, because of its unusual air breathing organ and its popularity in the aquarium trade, is the Anabantid fishes, which includes the bettas (Siamese fighting fish) and gouramis. The embryonic stage of most Anabantid fishes is relatively short, with hatching occurring 12 to 24 h post-fertilization (hpf) (Das, 1927; Hisoaka and Firlit, 1962; Munshi and Hughes, 1992). Larvae are small (<4 mm) and buoyant, and are maintained in the bubble nest or float at the surface for the first few days. After the large yolk sac is consumed, the larvae begin actively searching for food sources outside of the bubble nest (Picciolo, 1964; Munshi and Hughes, 1992).

Respiration of early teleost larvae is via cutaneous diffusion, replaced later by active branchial respiration as the gills develop (Hughes et al., 1986; Burggren, 1993; Rombough, 2002, 2007). For example, in the climbing perch (Anabas testudineus), an air breathing Anabantid, gills begin to differentiate by 24 hpf and become functional by
39 hpf (Hughes et al., 1986), but the exact function of the gills at this early stage is unclear. Rombough (2002, 2007) has suggested that the gills of developing teleost larvae may serve ionoregulatory functions well before they are employed for gas exchange. The gills of air breathing fishes, including Anabantids, differ morphologically from those of completely aquatic fishes. There are generally fewer and smaller secondary lamellae, and the overall size and effective surface area of the third and fourth gill arches (GA-V and GA-VI) are reduced (Figure 2.1; Prasad, 1988; Munshi and Hughes, 1992; Huang et al., 2007). Many of these fish are obligatory air breathers, at least as adults, as they are unable to meet their oxygen demands through aquatic respiration alone (Kramer, 1983; Graham, 1997). Additionally, the major vessels of GA-V and GA-VI may serve as blood shunts, which could prevent branchial oxygen loss in hypoxic water (Olson et al., 1986; Graham, 1997). The blood-water diffusion distance at the secondary lamellae increases with body size, and may be a contributing factor in the onset of air breathing (Mishra and Singh, 1979; Hughes et al., 1986). For example, a 4-fold increase in this distance is seen in A. testudineus between the completely aquatic phase (1-2 \( \mu \text{m} \)) and the bimodal phase (4-8 \( \mu \text{m} \)) (Mishra and Singh, 1979).

Early development of the labyrinth organ of Anabantid fishes generally occurs when larvae attain a total body length of 10-12 mm, usually 18-20 days post-fertilization (dpf) (Das, 1927), although its differentiation in A. testudineus has been recorded as early as at 60 hpf (Hughes et al., 1986). This structure develops out of the epibranhial segment of the first gill arch (GA-III) (Hughes and Munshi, 1973; Munshi et al., 1986). Though the labyrinth becomes highly convoluted as development proceeds, this organ is nonetheless continuous and is covered with a thin and highly vascularized respiratory
epithelium (Das, 1927; Bader, 1937; Peters, 1978). This vascularization of the labyrinth and surrounding suprabranchial chamber may allow these structures to take part in aquatic respiration prior to the onset of air breathing (Hughes et al., 1986). The plates of the labyrinth were once thought to be modified gills, but the pillar cells characteristic of gills are lacking in this structure (Hughes and Munshi, 1968), suggesting a non-branchial origin. Prior to the onset of air breathing, the diffusion distance of the labyrinth organ is greater than that of the secondary lamellae, but decreases with further growth of the larvae (Hughes et al., 1986). The growth of larvae immediately prior to the onset of air breathing owes mostly to rapid increases in girth, rather than length (Mishra and Singh, 1979). This decreased surface-to-volume ratio likely serves as a further impetus for the transition to bimodal respiration.

The purpose of this study was to explore morphological development of an Anabantid, the blue gourami (Trichogaster trichopterus) developing in normoxic conditions, with special reference to the respiratory system. Whole body measurements (length, mass, cutaneous surface area) were made for larvae of various stages, as were observations of the development and growth patterns of branchial organs (gills, labyrinth). These data will permit a better understanding of this species’ respiratory development, and will serve as a basis for determining the morphological implications of the subsequent experimental manipulations on its ontogeny, as reported in Chapter 3.
2.2. Materials and Methods

Larvae were reared as described in Chapter 1 (Section 1.2.2. Care and Maintenance of Larvae). Food was withheld from experimental animals for 18 to 24 h prior to making any morphological measurements.

2.2.1. Whole Body Measurements

From fertilization through 90 dpf, developing gouramis were randomly selected on a daily (1-14 dpf) to weekly (15-90 dpf) basis, and were euthanized with an overdose (>250 mg · l⁻¹) of Tricane (MS-222) (American Veterinary Medical Association, 2001). Light microscopy (Nikon Eclipse E200 compound microscope), captured via a Javelin digital camera and analyzed with ImagePro (Media Cybernetics) software, was employed to measure total body length (L_{TB}; ±0.1 mm) of gourami larvae. Larvae were then placed on pre-massed squares of aluminum 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 massed in groups, with 10-20 animals per group, and the average larval mass calculated. Older larvae and juveniles (15-90 dpf) were massed individually. Total body length of juveniles was measured with a caliper from snout to fork of tail. Juveniles, whose body form is dorso-ventrally flattened, were traced twice, once on each side of the body, and the outlines were measured with ImagePro to determine cutaneous surface area (SA_C; ±1 mm²).
2.2.2. Branchial Measurements

Larvae and juveniles to be dissected for morphological observations of the gills and labyrinth were also euthanized, and measurements of total body length, total body wet mass, and cutaneous surface area were recorded. Animals were then placed in 10% neutral buffered formalin for less than 24 h, so as to minimize shrinkage while sufficiently hardening the tissues in preparation for dissection. After this time, the opercula were removed, and gills and labyrinth organs were extracted and immersed in 1% Alcian blue for ~5 s to allow for easier microscopic observation of respiratory structures. Measurements of gills followed that of Hughes (1966, 1984), and included gill arch lengths, selected filament lengths, number of filaments per arch, number of secondary lamellae per selected filament, and surface area of secondary lamellae (Figure 2.2). Each measurement was made for the gills on both sides of the body, and the final recorded measurement represents an average.

All morphological measurements of the gills were obtained using ImagePro image analysis software. Filaments and secondary lamellae were quantified by dividing each gill arch 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. Measurements were representative of that third of the gill arch. Filament density was calculated as number of filaments per gill arch length (filaments · mm⁻¹), while lamellar density refers to the number of secondary lamellae per filament length (lamellae · mm⁻¹). For each gill arch, surface area estimations of single secondary lamellae (SA<sub>SL</sub>) were obtained by multiplying the length and width of several lamellae of selected filaments, and then
multiplying X 2 to account for the top and bottom of the lamellae. Lamellar surface area (SA_L) was calculated by determining the lamellar surface area of each gill arch from surface area measurements of individual lamellae as represented in Equation 2.1.

**Equation 2.1.** \[ SA_L = (SA_{SL}) \times (\text{lamellae per filament}) \times (\text{filaments per gill arch}) \]

SA_L was then summed for all four gill arches, and this value multiplied X 2 to account for the gills on both sides of the fish, or total lamellar surface area (SA_{TL}; mm^2).

Labyrinth surface area (SA_{Lb}) was estimated by tracing the outer circumference of the labyrinth organ, as viewed in several images taken from different angles. The area was calculated from this circumference using ImagePro, and was multiplied X 2 to account for both sides of the labyrinth. Total labyrinth surface area was calculated by multiplying the original measurement X 2 to account for both labyrinth organs.

Total respiratory surface area (SA_{TR}) was estimated by summing total lamellar, labyrinth and cutaneous surface areas.

Gourami body size and the relative growth of respiratory organs become highly variable as development progresses (Figure 2.3). Thus, rather than comparing morphometric development of respiratory structures on the basis of chronological age, measurements were typically expressed relative to total body wet mass.

### 2.2.3. Statistical Analyses

Data were analyzed for statistically significant differences throughout chronological development (dpf) using either One-Way ANOVA with post-hoc tests (e.g. Holm-Sidak Multiple Comparisons Procedure), or Kruskal-Wallis One-Way ANOVA on Ranks, when a non-parametric test was required. Regression analysis was employed
when data were plotted against total body wet mass. For all statistical tests, significance was declared if \( p < 0.05 \). All statistical analyses were performed with SigmaStat (Systat) software.

2.3. Results

2.3.1. Whole Body Measurements

Total body length \( (L_{TB}) \) significantly \( (p < 0.001) \) increased from \(~3\) mm at hatching to \(~20\) mm by \(90\) dpf, and is best described by a linear relationship (Table 2.2; Figure 2.4-A). Total body wet mass \( (M_{TB}) \) also increased significantly \( (p < 0.001) \) from \(~0.1\) mg at hatching to \(~130\) mg by \(90\) dpf, and is best described by a quadratic equation (Table 2.2; Figure 2.4-B). Total body length significantly increased in a biphasic manner when analyzed as a function of body mass \( (p < 0.001) \) (Table 2.2; Figure 2.5), as determined by maximized \( r^2 \) values when regression lines were summed. Total body length increased rapidly between hatching and \(~12\) mg, and then the rate of increase slowed with further increase in body mass.

When cutaneous surface area \( (SAC) \) of juvenile blue gourami was analyzed as a function of development (Figure 2.6-A), no significance was found. However, when \( SAC \) was analyzed as a function of body mass (Figure 2.6-B), a significant \( (p < 0.001) \) positive relationship was revealed, with \( SAC \) increasing more than 5-fold over the range of body mass observed.
2.3.2. Gill Development

The lengths of all gill arches ($L_{GA}$) showed a significant ($p<0.001$) positive relationship with body mass, increasing ~70% on average over the range of body mass observed (Figure 2.7-A). Filament lengths ($L_F$) of all four gill arches showed a significant ($p<0.001$) positive relationship with body mass at GA-III, GA-IV, and GA-V, with $L_F$ increasing ~80% on average between these gill arches. No significant increase in $L_F$ was noted at GA-VI.

The number of filaments at each gill arch (filament count, $C_F$; Figure 2.8-A) and the total number of filaments per animal (total filament count, $C_{TF}$; Figure 2.8-B) were analyzed as functions of body mass. The number of filaments increased significantly ($p<0.001$) at GA-III, GA-IV and GA-V, nearly doubling on average over the developmental period observed. No significant increase in $C_F$ occurred at GA-VI. $C_{TF}$ also increased significantly over the range of body mass observed ($p<0.001$), from 322 to 468 filaments.

When filament density ($D_F$) was analyzed as a function of body mass, there was a trend towards decreasing density as body mass increased (Figure 2.9). A significant negative relationship was found at GA-III ($p=0.009$) and GA-VI ($p=0.028$), decreasing ~10% on average for these gill arches. No significance was found at either GA-IV ($22\pm0.3$ filaments · mm$^{-1}$) or GA-V ($D_F = 24\pm0.5$ filaments · mm$^{-1}$).

The total number of secondary lamellae per gill arch (lamellar count, $C_L$) was also analyzed as a function of body mass (Figure 2.10-A). A significant ($p<0.001$) positive relationship was observed at GA-III, GA-IV, and GA-V, with $C_L$ about doubling on
average, but no significance occurred at GA-VI (193±71.4 lamellae). When total body lamellar count (C\textsubscript{TL}) was analyzed as a function of body mass (Figure 2.10-B), there was a significant (p<0.001) positive relationship, with C\textsubscript{TL} more than tripling, from 2712 to 9012 lamellae, over the body mass range studied.

There was a trend towards decreasing lamellar density (D\textsubscript{L}) as a function of body mass (Figure 2.11). A significant negative relationship was found only for GA-IV (p=0.012), with D\textsubscript{L} decreasing ~15% over the range of body mass observed. No significant difference was observed for GA-III (70±2.4 lamellae · mm\textsuperscript{-1}), GA-V (64±1.6 lamellae · mm\textsuperscript{-1}), or GA-VI (57±3.9 lamellae · mm\textsuperscript{-1}).

There was a significant positive relationship of single lamellar surface area (SA\textsubscript{SL}) as a function of body mass (Figure 2.12-A) at all gill arches (GA-III, GA-IV, GA-V: p<0.001; GA-VI: p=0.031), with SA\textsubscript{SL} increasing ~50% on average. There was also a significant (p<0.001) positive relationship of lamellar surface area by gill arch (SA\textsubscript{L}) as a function of body mass (Figure 2.12-B) at GA-III, GA-IV, and GA-V, with SA\textsubscript{L} of these gill arches more than tripling on average. No significant relationship was found at GA-VI. Total body lamellar surface area (SA\textsubscript{TL}) similarly had a significant (p<0.001) positive relationship as a function of body mass (Figure 2.13), with SA\textsubscript{TL} increasing more than 4-fold over the range of body mass studied.

2.3.4. Labyrinth Organ Development

Total labyrinth surface area (SA\textsubscript{Lb}) analyzed as a function of body mass revealed a significant (p<0.001) positive relationship, with SA\textsubscript{Lb} increasing more than 30-fold, from 0.15 mm\textsuperscript{2} to 4.8 mm\textsuperscript{2}, over the range of body mass observed (Figure 2.14).
2.3.5. Total Respiratory Surface Area

When cutaneous, lamellar, and labyrinth surface areas were analyzed as functions of body mass, a significant (p<0.001) positive relationship was observed for each respiratory structure (Figure 2.15-A), as stated previously. Cutaneous surface area increased over the range of body mass observed, but its value as a percent $SA_{TR}$ did not change (~75%). Total lamellar surface area similarly increased over the range of body mass studied, and the corresponding percent $SA_{TR}$ also did not change (~23%). Total labyrinth surface area, however, showed both an increase in raw value and as a percent $SA_{TR}$ (0.5% to 1.6%). Total respiratory surface area ($SA_{TR}=SA_C+SA_{TL}+SA_{Lb}$) plotted against body mass yielded a significant positive relationship (p<0.001), with $SA_{TR}$ more than tripling over the range of body mass studied (Figure 2.15-B). However, when mass-specific cutaneous, lamellar, and labyrinth surface areas (mm$^2$ g$^{-1}$) were analyzed as functions of body mass, the trend differed for each structure (Figure 2.16). Mass-specific $SA_C$ and mass-specific $SA_{TL}$ declined by ~40% and ~30%, respectively, while mass-specific $SA_{Lb}$ increased by more than 80% over the range of body mass observed.
2.4. Discussion

2.4.1. Whole Animal Size and Shape

Blue gourami hatch at ~24 h post-fertilization (Hisaoka and Firlit, 1962; Degani, 2001). At this time larvae are quite small, having a total body length ~2 mm and total body wet mass ~2 mg (Degani, 1991). This is similar to other Anabantid species; for example, the climbing perch *Anabas testudineus*, which hatches at 10 hpf, is only 2.1 mm and 0.11 mg (Hughes *et al*., 1986). The yolk sac provides all nutritional needs for the first few days post-hatch, during which time larvae are maintained in the bubble nest by the adult male. While the gills may start differentiating during this time, cutaneous respiration dominates, as the skin is thin and well vascularized (Hughes *et al*., 1986).

Early larval growth is mostly represented by increased body length ($L_{TB}$; Figures 2.4-A, 2.5), but by a body mass of ~12 mg the rate of $L_{TB}$ increase slows (Figures 2.5). This body mass coincides with a $L_{TB}$ of 10-12 mm, which is the point in development that most Anabants begin air breathing (Das, 1927; Munshi and Hughes, 1992). These findings are consistent with those of Mishra and Singh (1979), in which the girth of developing *A. testudineus* increased more rapidly than $L_{TB}$ immediately prior to the onset of air breathing.

Cutaneous surface area ($SA_{C}$) increased over the range of body mass (Figure 2.6-B), but the mass-specific $SA_{C}$ ($mm^2 \cdot g^{-1}$) declined (Figure 2.17). Comparisons of developing blue gourami ~100 mg reveal a mass-specific $SA_{C}$ (~1400 $mm^2 \cdot g^{-1}$) close to that found for similarly-sized larval walleye (*Stizostedion vitreum*; Rombough and Moroz, 1997), salmon (*Salmo salar*; Wells and Pinder, 1996) and carp (*Cyprinus carpio*; Oikawa and Itazawa, 1985), all of which had a mass-specific $SA_{C}$ of ~1500 $mm^2 \cdot g^{-1}$.
For both salmon and walleye, $S_{AC}$ accounted for >95% of the total respiratory surface area ($S_{ATR}$) available in newly hatched larvae (up to ~60 mg; Wells and Pinder, 1996; Rombough and Moroz, 1997), while this value was only ~77% for blue gourami larvae below this body mass. These are the first data detailing the $S_{AC}$ of any developing air breathing fish. However, Munshi et al. (1980) report that the mass-specific $S_{AC}$ for adult *Clarias batrachus* (100 g), an air breathing fish of the Family Clariidae, is 173 mm$^2$ · g$^{-1}$.

If the equation representing the relationship between $S_{AC}$ and body mass is used to estimate the value for *C. batrachus* at 100 mg, the result is ~1020 mm$^2$. This value is quite lower than that found for developing blue gourami, and is possibly because the relative $S_{AC}$ of larger fish has a smaller negative slope with increasing body mass than that of larvae and juveniles.

### 2.4.2. Gill Development

Although the telost gill is best known as a gas exchange organ, it also serves a number of other functions in both developing and adult animals. Some of these include ionoregulation, osmoregulation, acid-base balance and ammonia excretion (Rombough, 2007). In adult fishes a major role of the gill arches and primary filaments is that of ion exchange (Laurent, 1984), and in developing animals it has been argued that gills develop relatively early, and are needed for ionoregulation well before they are required for respiration (Rombough 2002, 2007). Gill arches and filaments begin to differentiate in the Anabantid *A. testudineus* at 24 h post-fertilization, but secondary lamellae do not develop until 4 dpf (Hughes et al., 1986). However, the need for branchial gas exchange in larvae and juveniles may occur prior to the development of the secondary
lamellae, and thus gill arches and filaments might play a role in gas exchange until gills mature (Rombough, 2002). Lamellar gas exchange can be influenced by a number of factors, including the respiratory surface area, proportion of blood flow perfusing the lamellae, thickness of the blood-water diffusion barrier, and oxygen capacity and binding affinity of hemoglobin (McDonald and McMahon, 1977; Evans et al., 2005).

In agreement with the data presented here for larval and juvenile blue gourami (Figure 2.7), GA-III and GA-IV in other Anabantids are larger and more well developed than are GA-V and GA-VI (Das, 1927; Prasad, 1988; Maina, 2002), suggesting their importance in aquatic gas exchange. Moreover, the efferent branchial arteries of these first two gill arches supply blood to the labyrinth organ (Burggren, 1979; Munshi and Hughes, 1992; Graham, 1997), and are thus also integral to aerial respiration. Additionally, it has been reported that GA-III and GA-IV are responsible for ionoregulation in *Trichogaster leeri*, as the number of mitochondria-rich (MR) cells increased in these arches upon exposure to ionic stress, while there was no difference found in the number of MR cells of GA-V and GA-VI (Huang et al., 2007). The importance of the first two gill arches in gas exchange and ionoregulation help to explain the rapid growth rate of GA-III and GA-IV in developing blue gourami (Figure 2.7-A), but less well understood is the development of GA-V, which showed the fastest rate of growth, despite evidence that a major role of GA-V and GA-VI is that of a blood shunt. For example, studies of Anabantids have found large-bore anterior-arterial shunts in GA-V and GA-VI (Olsen et al., 1986; Huang et al., 2007), suggesting that these arches are specialized for the transport of oxygenated blood to the body. This is
likely an adaptation to living in hypoxic environments, wherein oxygen can readily be lost across gills with a large surface area (Burggren, 1979; Munshi and Hughes, 1992).

Filaments of GA-III, GA-IV, and GA-V lengthened (Figure 2.7-B) and increased in number (Figure 2.8-A) with increased body mass, but not necessarily all at the same rate. Again, the importance of GA-III and GA-IV as respiratory and ionoregulatory structures explains the rapid filament proliferation and growth on these two arches, but the significant increase of filament length and number on GA-V requires more investigation. Perhaps GA-V is more plastic in its development than GA-VI, which has been reported to be less responsive to environmental stress (Huang et al., 2007). GA-VI did not show a significant increase in \( L_F \) or \( C_F \), which is likely indicative of its fixed role as a blood shunt. As developing gouramis generally are exposed to varying levels of hypoxia in nature, the lack of exposure during this study may have shifted the development of GA-V to more traditional gill functions (e.g. ion regulation and gas exchange), rather than to that of a blood shunt.

Filament density (\( D_F \)) at GA-III and GA-VI decreased as larvae grew larger, but no change was observed at GA-IV or GA-V (Figure 2.9). After initial differentiation of the gill arches, new filaments develop only at the growing end of the arch. Thus the number of filaments developing at any point along an arch is fixed, and further growth of the gill arch only increases the distance between the existing filaments (i.e. decreases \( D_F \)). Therefore, the decreased filament density at GA-III and GA-VI likely owes to the significant increase in \( L_{GA} \) at these arches (Figure 2.7-A). The lack of a significant decrease in \( D_F \) at GA-IV and GA-V is confounding, as \( L_{GA} \) increased at these arches as well. However, the trend of decreasing \( D_F \) suggests that significance may have been
determined if a larger range of body mass, and thus a larger sample size, was observed.

Secondary lamellae generally form on filaments around 4 dpf in Anabantids (Hughes et al., 1986; Prasad, 1988). The blood-water diffusion distance of these structures is very thin (1-2 µm), and their large numbers increase the surface area of the gills tremendously, allowing for efficient branchial gas exchange as the need arises in developing fishes.

The significant increases in number of lamellae at GA-III, GA-IV and GA-V (Figure 2.10-A) and overall (Figure 2.10-B) were expected, as the increase in number and length of filaments was more than enough to counter the slight decrease in filament density (~0.02 filaments · mg⁻¹; Figure 2.9) and lamellar density (~0.06 lamellae · mg⁻¹; Figure 2.12). However, compared with the lamellar numbers found in completely aquatic teleosts, those of air breathing fishes are much reduced, likely due to the decreased importance of branchial respiration as bimodality develops (Munshi and Hughes, 1992), and of the role of GA-V and GA-VI as blood shunts.

Lamellar density (Dₗ) is generally lower for air breathing fishes than for aquatic species (Munshi and Hughes, 1992), and generally decreases with increasing body size in Anabantid fishes. This reduced density increases the distance between secondary lamellae, and thus decreases the resistance to water flow across the gills (Munshi and Hughes, 1992). In the aquatic phase of larval A. testudineus, for example, secondary lamellae are 0.009 mm apart, while that distance increases to 0.012 mm in the bimodal phase (Prasad, 1988). In developing gourami, however, lamellar density remained relatively stable over the size range observed, with only that of GA-IV showing a
significant decline (Figure 2.11). As with filaments, the nature of lamellar growth is that new lamellae develop only at the growing tip of the filament; thus further overall lengthening of the filament results in more lamellae at the tip, but existing lamellae simply grow further apart (i.e. reduced D_L). Perhaps again it was the relatively small range of body mass observed that precluded significance being determined at all four arches, or that all of the animals measured had already made the transition to bimodal respiration, and thus the major decline in D_L had already occurred. In the case of the latter, D_L at GA-III for the gouramis observed here (~70 lamellae · mm⁻¹) was similar to that reported for bimodal *A. testudineus* (75 lamellae · mm⁻¹; Prasad, 1988), while that at GA-V and GA-VI was slightly lower (~57-64 · lamellae mm⁻¹).

Single lamellar surface area (SA_SL; Figure 2.12-A) and lamellar surface area by gill arch (SA_L; Figure 2.12-B) increased significantly at GA-III, GA-IV, and GA-V throughout the range of body mass observed. Again, this increase was expected for GA-III and GA-IV due to their importance in respiration and ionoregulation. As GA-V has been reported to function as a blood shunt, it was not expected to show the increased SA_L that it did, but the increased L_GA and L_F observed would make this a reasonable finding. The purpose for such growth, as mentioned previously, may be that GA-V is capable of functioning more like GA-III and GA-IV, and less like a blood shunt, when hypoxic conditions are not present. GA-VI did not show a significant increase in SA_L, which is what was expected due to its apparently fixed nature as a blood shunt.

Total lamellar surface area (SA_TL) increased across the range of body mass observed (Figure 2.13), as was expected, since the largest arches (GA-III and GA-IV) contributed most to the overall SA_TL. The increased gill area observed in *Colisa*
*fasciatus* was mainly a result of increased filament length throughout early development (Prasad, 1988). This explanation would also be reasonable for developing blue gourami, as the gill arches which showed significant increases in SA<sub>TL</sub> (i.e. GA-III, GA-IV, and GA-V) were the same as those observed to have significant increases in filament length (Figure 2.8).

Total lamellar surface area of developing carp (*C. carpio*; 100 mg) is ~80 mm<sup>2</sup> (Oikawa and Itzawa, 1985), and for walleye (*S. vitreum*; 100 mg) the value is ~85 mm<sup>2</sup> (Rombough and Moroz, 1997), while the developing Atlantic salmon (*S. salar*; 100 mg) has a SA<sub>TL</sub> of ~55 mm<sup>2</sup> (Wells and Pinder, 1996). These values were quite a bit higher than that observed for similarly-sized blue gourami (37.9 mm<sup>2</sup>; Figure 2.14), and lends credence to the general observation that completely aquatic fish tend to have a greater gill area than do air breathers (Palzenberger and Pohla, 1992). However, the only comparable developmental study for an air breathing fish of similar size reported a SA<sub>TL</sub> of ~75 mm<sup>2</sup> for the Anabantid *Colisa fasciatus* (100 mg; Prasad, 1988), which is nearly twice that observed for the blue gourami. While the blue gourami is an obligatory airbreather, the respiratory status of *C. fasciatus* is debatable; Prasad and Singh (1984) list this species as obligatory, while Ojha et al. (1977) describe it as non-obligatory. This incongruity may suggest that the gills of *C. fasciata* are better developed than more definitive obligatory air breathers such as the blue gourami, and would help to explain the discrepancy in SA<sub>TL</sub> between these two species. Other studies of larger air breathers (1 g) reveal SA<sub>TL</sub> ranging from 93 mm<sup>2</sup> (*B. boddarti*; Hughes and Al-Kadhomy, 1986) to 616 mm<sup>2</sup> (*R. strigosa*; Santos et al., 1994), with the Anabantid *A. testudineus* having a SA<sub>TL</sub> of 278 mm<sup>2</sup> (Hughes et al., 1973). If the equation for the
SA_{TL} of developing blue gourami is extrapolated to animals weighing 1 g, the value would be \(~330 \text{ mm}^2\). Caution must be taken in making this comparison, however, as allometric scaling often changes with development (e.g. Oikawa and Itazawa, 1985; Prasad, 1988; Wells and Pinder, 1996). Nevertheless, the range of values given here for *T. trichopterus* (Figure 2.16) is within reason given that presented in other studies.

Allometric scaling can reveal patterns of growth and is a useful tool in comparing inter- and intra-specific studies. The general equation is \( y=am^b \). Here, \( y \) is SA_{TL}; \( a \) is a constant; \( m \) is body mass; and \( b \) is the mass exponent. It is the mass exponent that is usually used to make comparisons, as this value describes how the measurement in question scales with body mass. For developing blue gouramis in this study, \( b \) was 0.79. This is similar to that reported for developing *C. fasciatus* (0.80; Prasad, 1988), juvenile *C. carpio* (0.79; Oikawa and Itazawa, 1985), and adults (100 g) of the air breathing fishes *Clarias batrachus* (0.78; Hughes and Munshi, 1979), *Channa striatus* (0.72; Munshi, 1985), and *Heteropneustes fossilis* (0.75; Hughes and Munshi, 1979). The scaling exponent for the adult (100 g) Anabantid *A. testudineus* is only 0.62 (Hughes and Munshi, 1979), and represents the reduced surface area of the gills that corresponds with the obligatory lifestyle of this species. It is likely that adult blue gouramis, also obligatory air breathers, would have a similar low mass exponent.
2.4.3. Labyrinth Organ Development

The total surface area of the labyrinth organ ($SA_{Lb}$) of blue gouramis increased more than 30-fold throughout the developmental period observed (Figure 2.14). Although the present study did not determine the point in development that differentiation of the air breathing organ commenced, the smallest animal observed (~25 mg) had little more than a labyrinth “bud” (~0.15 mm$^2$), and thus it seems reasonable to assume that labyrinth development does not begin very much prior to this point.

Das (1927) reported that the first traces of the labyrinth organ in $A.\ testudineus$ and $Macropodus\ opercularis$ were not present until larvae reached a length of 12 mm, and Munshi and Hughes (1986) observed that even in six-month old (1 g) $A.\ testudineus$ the labyrinth organ is “not well differentiated, having only one saucer-shaped plate”. Hughes et al. (1986), however, report that labyrinth differentiation in $A.\ testudineus$ begins as early as 60 hpf. It may be that development of initial structures takes place early in Anabantid ontogeny, but that further development is suppressed until later in development. Bader (1937; Reviewed in Graham, 1997) hypothesized that lack of access to air affects differentiation of the air breathing organ in $M.\ opercularis$. Air access was denied to 12 to 18 mm larvae for one to seven months. Throughout this period, histological samples revealed that the vascular lining of the suprabranchial chamber (SBC) of experimental fish remained largely undifferentiated compared with that of control animals. After seven months, many of the experimental larvae were allowed access to air, at which time the air breathing behavior commenced and frequency of gill ventilation decreased. Further histological studies concluded that the larvae were able to “recover” from developing without air access, as vascularization of
the epithelia of the SBC increased when larvae were allowed access. These experiments suggest that some factor involved in aerial respiration acts as a necessary environmental stimulus for the differentiation of the labyrinth organ and SBC.

Hughes et al. (1973) reported the labyrinth surface area of a 1 g A. testudineus to be 80.3 mm\(^2\). If the equation representing \(SA_{Lb}\) of developing blue gourami is extrapolated to include animals weighing 1 g, the \(SA_{Lb}\) would be only \(\approx 33\) mm\(^2\). However, this value, which is less than half that given for A. testudineus, is based on a linear regression equation determined for animals between 25 and 216 mg, and does not take into consideration possible changes in allometric scaling that may exist for smaller or larger animals. The nature of labyrinth organ growth would presumably preclude a completely linear increase in \(SA_{Lb}\). During the developmental period studied here, the labyrinth remains a relatively simple structure, consisting of a single "plate". As development progresses, however, the labyrinth gains more plates and each plate begins to curve as it elongates (Das, 1927; Bader, 1937; Munshi and Hughes, 1986), presumably adding almost exponentially to \(SA_{Lb}\). The increased \(SA_{Lb}\), along with a decreased blood-air barrier and aquatic ventilation frequency, likely enhances aerial oxygen uptake capabilities (Kramer, 1983). This will be discussed further in Chapter 3.

While no comparisons of the developing labyrinth organ of Anabantid fishes could be found, Hughes et al. (1973) reported the scaling exponent of the labyrinth of adult A. testudineus to be 0.78. This is quite lower than that found here for developing blue gouramis (1.33), and is indicative of the rapid growth of the air breathing organ during the transition to bimodal respiration.
2.4.4. Total Respiratory Surface Area

Total respiratory surface area (SA\textsubscript{TR}) in blue gourami revealed more than a five-fold increase during the developmental period observed (Figure 2.15-B). While each structure measured also showed significant increases in gross area (Figure 2.15-A), the mass-specific SA\textsubscript{C} and mass-specific SA\textsubscript{TL} decreased by 41% and 28%, respectively, while the mass-specific SA\textsubscript{Lb} nearly doubled (Figure 2.16). While no similar data could be found for other air breathing fish to serve as a comparison, in 1 d post-hatch walleye (\textit{S. vitreum}), SA\textsubscript{C} accounts for 99.9% (8500 mm\textsuperscript{2} \cdot g\textsuperscript{-1}) of SA\textsubscript{TR}, with the gills making up the other 0.01% (~5 mm\textsuperscript{2} \cdot g\textsuperscript{-1}; Rombough and Moroz, 1997). By 200 mg body mass, however, the mass-specific SA\textsubscript{C} has decreased to ~1500 mm\textsuperscript{2} \cdot g\textsuperscript{-1} (~58% SA\textsubscript{TR}), while the mass-specific SA\textsubscript{TL} has increased to 1100 mm\textsuperscript{2} \cdot g\textsuperscript{-1} (~42% SA\textsubscript{TR}). Prasad (1988) has suggested that, for air breathing fish, it is at the change to the bimodal phase (i.e. onset of air breathing) that the gill area ceases to increase with body size. The absence of an increase in mass-specific SA\textsubscript{TL} in the blue gourami may be due simply to the lack of branchial measurements prior to the onset of air breathing. The mass-specific rates of both the skin and gills of the walleye decline in animals larger than ~200 mg, but the slower decline of mass-specific SA\textsubscript{TL} means that the gills overtake the mass-specific SA\textsubscript{C} at ~700 mg in this species (Rombough and Moroz, 1997). In the carp (\textit{C. carpio}), mass-specific SA\textsubscript{TL} overtakes mass-specific SA\textsubscript{C} at ~350 mg, and it is at this point that rate of mass-specific SA\textsubscript{TL} also begins to slow (Oikawa and Itazawa, 1985). A similar phenomenon can be seen in developing blue gouramis, in which the slower decline of mass-specific SA\textsubscript{TL} indicates that, if the data in Figure 2.16 are extrapolated, mass-specific SA\textsubscript{TL} will overtake mass-specific SA\textsubscript{C} at a body mass of ~350 mg (Figure 2.17).
Although mass-specific $SA_{lb}$ nearly doubled over the developmental period observed, by 216 mg it still only represented ~1.6% of $SA_{TR}$. As air breathing has already become established by this point in development (See Chapter 3), it may be that, because of the thickening of the skin and the formation of scales during the larval-to-juvenile transition, $SA_c$ does not play a major role in gas exchange, and that $SA_{TR}$ may be better represented by $SA_{TL}$ and $SA_{lb}$. It may also be that the highly vascularized lining of the SBC plays a crucial role in early aerial oxygen uptake, and that the air breathing organ of developing Anabantids should be considered to be the entire SBC and its contents, and not the labyrinth organ alone.

2.5. Summary

The purpose of this study was to explore morphological development of an air breathing fish, the blue gourami, developing in normoxic conditions. Whole body measurements, including total body length, total body wet mass, and cutaneous surface area, increased significantly over the developmental period studied. Various aspects of respiratory morphology also significantly increased over this period, including total body lamellar surface area, although there was a general lack of significant growth at GA-VI, which is indicative of this arch’s role as a blood shunt in Anabantid fishes. Also showing significant increases were labyrinth organ surface area and total respiratory surface area. While the mass-specific cutaneous and lamellar surface areas ($mm^2 \cdot g^{-1}$ total body wet mass) declined throughout development, the mass-specific labyrinth surface area increased, suggesting its escalating importance with continued growth of the animal, and with its transition to air breathing.
Table 2.1. Abbreviations of morphological parameters studied.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;F&lt;/sub&gt;/C&lt;sub&gt;TF&lt;/sub&gt;</td>
<td>Filament count by gill arch/Total body filament count</td>
</tr>
<tr>
<td>C&lt;sub&gt;L&lt;/sub&gt;/C&lt;sub&gt;TL&lt;/sub&gt;</td>
<td>Lamellar count by gill arch/Total body lamellar count</td>
</tr>
<tr>
<td>D&lt;sub&gt;F&lt;/sub&gt;</td>
<td>Filament density (filaments · mm&lt;sup&gt;-1&lt;/sup&gt; gill arch length)</td>
</tr>
<tr>
<td>D&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Lamellar density (secondary lamellae · mm&lt;sup&gt;-1&lt;/sup&gt; filament length)</td>
</tr>
<tr>
<td>L&lt;sub&gt;F&lt;/sub&gt;</td>
<td>Filament length (mm)</td>
</tr>
<tr>
<td>L&lt;sub&gt;GA&lt;/sub&gt;</td>
<td>Gill arch length (mm)</td>
</tr>
<tr>
<td>L&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>Total body length (mm)</td>
</tr>
<tr>
<td>S&lt;sub&gt;A&lt;/sub&gt;C</td>
<td>Cutaneous surface area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>S&lt;sub&gt;A&lt;/sub&gt;SL/S&lt;sub&gt;A&lt;/sub&gt;L</td>
<td>Single lamellar surface area/Lamellar surface area by gill arch (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>S&lt;sub&gt;A&lt;/sub&gt;TL</td>
<td>Total lamellar surface area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>S&lt;sub&gt;A&lt;/sub&gt;Lb</td>
<td>Total labyrinth surface area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>S&lt;sub&gt;A&lt;/sub&gt;TR</td>
<td>Total respiratory surface area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>Total body wet mass (mg)</td>
</tr>
</tbody>
</table>
Table 2.2. Summary statistics for whole body measurements of gouramis reared in normoxia through 90 dpf. See Table 2.1 for list of abbreviations. NS: No significance.

<table>
<thead>
<tr>
<th>Rearing Group</th>
<th>( L_{TB} )</th>
<th>( M_{TB} )</th>
<th>( S_{A_C} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Av. body mass:</td>
<td>( Y=1.74+0.20 ) dpf</td>
<td>( Y=3.27-0.72 ) dpf+0.03 dpf (^2)</td>
<td>( Y=107.9+13.72 ) mm (^2)</td>
</tr>
<tr>
<td>94±14.6 mg</td>
<td>( r^2=0.784; n=333 ) p&lt;0.001</td>
<td>( r^2=0.466; n=215 ) p&lt;0.001</td>
<td>n=14</td>
</tr>
<tr>
<td></td>
<td>( Y=4.15+0.707 M_{TB} ) ( r^2=0.761; n=142 ) p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( Y=10.68+0.066 M_{TB} ) ( r^2=0.859; n=74 ) p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( M_{TB}\leq 12 ) mg:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( M_{TB}&gt;12 ) mg:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: \( Y \) represents the dependent variable, and \( M_{TB} \) represents the independent variable.
Table 2.2. Summary statistics for gill measurements of gouramis reared in normoxia through 90 dpf. See Table 2.1 for list of abbreviations. III-VI indicates gill arch position. NS: No significance.

<table>
<thead>
<tr>
<th>Rearing Group</th>
<th>L&lt;sub&gt;GA&lt;/sub&gt;</th>
<th>L&lt;sub&gt;F&lt;/sub&gt;</th>
<th>C&lt;sub&gt;F&lt;/sub&gt;</th>
<th>D&lt;sub&gt;F&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Av. body mass:</strong></td>
<td>94±14.6 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III: Y=1.93+0.009 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>III: Y=0.185+0.001 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>III: 46+0.2 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>III: Y=23-0.02 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>r²=0.914; n=17</td>
<td>r²=0.827; n=17</td>
<td>r²=0.872; n=17</td>
<td>r²=0.371; n=17</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p=0.009</td>
<td></td>
</tr>
<tr>
<td>IV: Y=1.52+0.008 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>IV: Y=0.194+0.001 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>IV: 38+0.1 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>IV: Y=22±0.3</td>
<td></td>
</tr>
<tr>
<td>r²=0.915; n=17</td>
<td>r²=0.840; n=17</td>
<td>r²=0.756; n=17</td>
<td>n=17; (NS)</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>V: Y=24±0.5 filaments</td>
<td></td>
</tr>
<tr>
<td>V: Y=1.06+0.011 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>V: Y=0.15+0.0005 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>V: 31+0.1 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>V: Y=25-0.02 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>r²=0.858; n=17</td>
<td>r²=0.685; n=17</td>
<td>r²=0.713; n=17</td>
<td>r²=0.580; n=8</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p=0.028</td>
<td></td>
</tr>
<tr>
<td>VI: Y=1.09+0.003 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>VI: Y=0.11±0.03 mm</td>
<td>VI: 32±1.4 filaments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r²=0.687; n=12</td>
<td>n=8; (NS)</td>
<td>n=9; (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>L&lt;sub&gt;C&lt;/sub&gt;</th>
<th>D&lt;sub&gt;L&lt;/sub&gt;</th>
<th>SA&lt;sub&gt;SL&lt;/sub&gt;</th>
<th>SA&lt;sub&gt;L&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>III: Y=606.3+7.41 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>III: Y=69.4+2.43 mm</td>
<td>III: Y=0.0036+2.3e-4 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>III: Y=1,65+0.074 M&lt;sub&gt;TB&lt;/sub&gt;</td>
</tr>
<tr>
<td>r²=0.898; n=17</td>
<td>n=17; (NS)</td>
<td>r²=0.880; n=11</td>
<td>r²=0.834; n=11</td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td></td>
<td>p&lt;0.001</td>
<td>p=0.001</td>
</tr>
<tr>
<td>IV: Y=499.0+4.82 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>IV: Y=70.2-0.06 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>IV: Y=0.0045+2.4e-4 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>IV: Y=1.48+0.059 M&lt;sub&gt;TB&lt;/sub&gt;</td>
</tr>
<tr>
<td>r²=0.352; n=17</td>
<td>r²=0.352; n=17</td>
<td>r²=0.836; n=11</td>
<td>r²=0.901; n=11</td>
</tr>
<tr>
<td>p=0.012</td>
<td>p=0.012</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>V: Y=275.9+2.90 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>V: Y=63.7+1.64 mm</td>
<td>V: Y=0.0037+0.0001 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>V: Y=0.96+0.020 M&lt;sub&gt;TB&lt;/sub&gt;</td>
</tr>
<tr>
<td>r²=0.859; n=17</td>
<td>n=17; (NS)</td>
<td>r²=0.748; n=11</td>
<td>r²=0.757; n=11</td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>(NS)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>VI: Y=193.0+31.9 mm</td>
<td>VI: Y=57.3+3.92 mm</td>
<td>VI: Y=0.0022+8.0e-5 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>VI: Y=0.63+0.189 mm²</td>
</tr>
<tr>
<td>n=5; (NS)</td>
<td>n=5; (NS)</td>
<td>r²=0.831; n=5</td>
<td>n=6; (NS)</td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p=0.031</td>
<td></td>
</tr>
<tr>
<td>SA&lt;sub&gt;TL&lt;/sub&gt;: Y=2864+30 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td>SA&lt;sub&gt;TL&lt;/sub&gt;: Y=5.72+0.322 M&lt;sub&gt;TB&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r²=0.906; n=17</td>
<td>r²=0.951; n=11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. Summary statistics for labyrinth surface area (SA\textsubscript{Lb}) and total respiratory surface area (SA\textsubscript{TR}) of gouramis reared in normoxia through 90 dpf. NS: No significance.

<table>
<thead>
<tr>
<th>Rearing Group</th>
<th>SA\textsubscript{Lb}</th>
<th>SA\textsubscript{TR}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>Y = -0.481 + 0.0219 M\textsubscript{TB}</td>
<td>Y = 40.5 + 1.19 M\textsubscript{TB}</td>
</tr>
<tr>
<td>Av. body mass:</td>
<td>r\textsuperscript{2} = 0.950; n=18</td>
<td>r\textsuperscript{2} = 0.986; n=8</td>
</tr>
<tr>
<td>94+14.6 mg</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>
Figure 2.1. Photographs of gill arches (GA) III-VI of a juvenile blue gourami (L_TB=15 mm) reared in normoxia. GA-III, from which the labyrinth organ (arrow) arises and GA-IV are larger and have longer, better developed filaments and more secondary lamellae than do GA-V and GA-VI.
Figure 2.2. Techniques for measurements of the gills of blue gourami larvae/juveniles. Arrows indicate measurements of (A) gill arch length; (B) filament length; (C) lamellar length; (D) lamellar width.
Figure 2.3. Branchial dissections of blue gourami juveniles (51 dpf) from the same brood reared in normoxia, showing the growth of the labyrinth organ (L). Gill arch III (GA) and total body lengths for each fish are also shown.
Figure 2.4. Total body length (A) and total body wet mass (B) as function of development of gouramis reared in normoxia through 90 dpf. Regression line with 95% confidence intervals (A) and quadratic function (B) shown. Statistics given in Table 2.2.
Figure 2.5. Total body length as function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression lines (in which $r^2$ values are maximized) with 95% confidence intervals are shown. Statistics given in Table 2.2.
Figure 2.6. Cutaneous surface area as function of (A) Development and (B) Total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression line with 95% confidence intervals shown where significance occurred; otherwise mean value shown. Statistics are given in Table 2.2.
Figure 2.7. Gill arch length (A) and filament length (B) as function of total body wet mass of gouramis reared in normoxia through 90 dpf. Regression lines shown. Statistics given in Table 2.3.
Figure 2.8. Number of filaments by gill arch (A) and total number of filaments per animal (B) as function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression lines shown (with 95% confidence intervals in B). Statistics given in Table 2.3.
Figure 2.9. Filament density ($D_F$) by gill arch as a function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression lines with 95% confidence intervals shown where significance occurred; otherwise mean±1 SE shown. Statistics given in Table 2.3.
Figure 2.10. Lamellar count by gill arch (A) and total body lamellar count (B) as function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression lines shown (with 95% confidence intervals in B); values in (B) are: $C_{L-III} + C_{L-IV} + C_{L-V} + 193.0$ (mean for $C_{L-VI}$, where $p>0.05$). Statistics given in Table 2.3.
Figure 2.11. Lamellar density by gill arch as function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression lines with 95% confidence intervals shown where significance occurred; otherwise mean±1 SE shown. Statistics given in Table 2.3.
Figure 2.12. Single lamellar surface area by gill arch (A) and total lamellar surface area by gill arch (B) as function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression lines shown. Statistics given in Table 2.3.
Figure 2.13. Total body lamellar surface area as function of total body wet mass for blue gouramis reared in normoxia through 90 dpf. Regression line with 95% confidence intervals shown. Statistics given in Table 2.3.
Figure 2.14. Total labyrinth surface area as a function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Regression line with 95% confidence intervals shown. Statistics given in Table 2.4.
Figure 2.15. (A) Respiratory surface area by structure, and (B) Total respiratory surface area ($SA_{TR}$) as function of total body wet mass (body mass) of blue gouramis reared in normoxia through 90 dpf. Regression lines shown (with 95% confidence intervals in B). Statistics given in Table 2.4.
Figure 2.16. Mass-specific respiratory surface area by structure as a function of total body wet mass of blue gouramis reared in normoxia through 90 dpf.
Figure 2.17. Extrapolations of mass-specific respiratory surface area by structure as a function of total body wet mass of blue gouramis reared in normoxia through 90 dpf. Slopes for each line given. Arrows indicate theoretical point in development that (A) Mass-specific SA_{TL} would overtake mass-specific SA_{C}, and (B) Mass-specific SA_{Lb} would overtake mass-specific SA_{TL}, according to extrapolations of existing data.
CHAPTER 3
PHYSIOLOGICAL DEVELOPMENT OF CARDIO-RESPIRATORY SYSTEMS
OF THE BLUE GOURAMI

3.1. Introduction

3.1.1. Cardio-Respiratory Ontogeny of Teleost Fishes

The physiology of adult teleost fishes has been well explored, as has that of completely aquatic developing fishes. However, few studies have examined the respiratory physiology of larval air breathing fishes. Respiration refers to a chain of events beginning with oxygen being taken up from the environment, use of this oxygen at mitochondria within individual cells, and the subsequent excretion of waste gases back out into the environment (Cech, 1990; Schmidt-Nielsen, 1997). Convective transport of oxygen via the cardiovascular system is an integral part of respiration in vertebrates.

The heart of embryonic fishes, like the heart of other vertebrate embryos, is the first functional organ, and the cardiovascular system the first functioning organ system (Burrgren and Pinder, 1991; Bagatto and Burrgren, 2006). The heartbeat of the blue gourami, for example, is established by 20 h post-fertilization (Hisaoka and Firlit, 1962). Embryonic and early larval fishes are capable of meeting all of their respiratory needs via diffusion (Rombough, 1988; Burrgren and Pinder, 1991; Pelster and Burrgren, 1996; Burrgren, 2005), and so the purpose of the embryonic heart beat remains elusive. It has been suggested that such action is required for angiogenesis, as the pulsatile pressure exerted by the heartbeat on the tips of the developing vasculature
result in proliferation of endothelial cells and thus growth of the vessels (Burggren, 2004, 2005). The heart rate of most, if not all vertebrate embryos increases at some point in ontogeny, and is likely due to changes in the intrinsic pacemaker during early stages, and neural and hormonal influences later in development (Adolph, 1968; Burggren and Warburton, 1994; Fritsche and Burggren, 1996; Barrionuevo and Burggren, 1999; Bagatto, 2005). For example, an increase in resting heart rate occurs in developing zebrafish (Danio rerio) between embryonic and early larval stages (Barrionuevo and Burggren, 1999; Bagatto, 2005), but no significant chronotropic responses to cholinergic or adrenergic agonists are present until 4 d post-fertilization (dpf) and 6 dpf, respectively (Bagatto, 2005). Additionally, it has been determined that, at least in normoxic conditions, conductive transport is not required in zebrafish larvae exposed until 14 dpf (Rombough, 2002).

While the secondary lamellae of the gills are the definitive sites of gas exchange in most adult fishes, the gills may serve other roles earlier in development, including those of ionoregulation, osmoregulation, chemoreception, acid-base balance and ammonia excretion (reviewed in Evans et al., 2005; Rombough, 2007). Gill arches and filaments form early in teleosts, at a time when larvae are still capable of obtaining all oxygen required via cutaneous diffusion (Rombough 1988; Burggren and Pinder, 1996). Thus, it has been suggested that the gills are required for ionoregulation prior to their necessity as respiratory structures (Rombough 2002, 2007). As fish grow larger, however, cutaneous respiration is no longer sufficient, and branchial respiration becomes crucial (Rombough, 2002; Jonz and Nurse, 2005). For example, zebrafish in
normoxia have a gill ventilation rate \( f_{GV} \) of ~3 beats \( \cdot \) min\(^{-1}\) at 3 dpf, but by 7 dpf, \( f_{GV} \) has increased to ~45 beats \( \cdot \) min\(^{-1}\) (Jonz and Nurse, 2005).

Oxygen consumption rates can reveal a great deal about an animal’s activity level and energetic costs, and can be indicative of subtle changes in physiological states that may otherwise go unnoticed (Cech, 1990). Oxygen consumption rates sharply increase around the time of hatching for a number of fishes, including zebrafish (Barrionuevo and Burggren, 1999), walleye (Rombough and Moroz, 1997), and the clownfish *Amphiprion melanopus* (Green, 2004). This may be due to an increase in spontaneous swimming (DeSilva *et al.*, 1986), or may be indicative of organogenesis or conversion of yolk to biomass (Barrionuevo and Burggren, 1999), and may actually represent a general trend in the development of all vertebrates.

3.1.2. Cardio-respiratory Ontogeny of Anabantids

As adults most Anabantid fishes are obligatory air breathers, having reduced gills that make meeting oxygen demands solely via aquatic respiration impossible, even in normoxic water (Prasad, 1988; Graham, 1997). Young larvae, however, are capable of obtaining sufficient oxygen from aquatic (i.e. cutaneous and branchial) respiration alone, despite high metabolic rates (Prasad, 1998). Mass-specific oxygen consumption rates of completely aquatic larvae have been reported for the Anabantid fishes *Colisa fasciatus* (Prasad and Singh, 1984; Prasad, 1989) and *Anabas testudineus* (Prasad, 1989), but no data on heart rate or gill ventilation rate could be found for any developing Anabantid fish.
Anabantids typically commence air breathing at 18 to 20 dpf, when they have attained a $L_{TB}$ of 10-12 mm (Das, 1927; Mishra and Singh, 1979; Prasad and Singh, 1984; Prasad, 1989). This behavior coincides with decreased cutaneous gas exchange resulting from the development of scales, and with an increase in the blood-water diffusion barrier of the gills, which results in decreased branchial oxygen uptake (Prasad, 1989). Reductions in aquatic oxygen consumption of 36% ($C.\ fasciatus$; Prasad and Singh, 1984) to 40% ($A.\ testudineus$; Prasad, 1989) have been reported for Anabantids once air breathing commences. While the respiratory physiology of the adult blue gourami has been studied (Burggren, 1979; Herbert and Wells, 2001), little is known of the physiology of developing gouramis. The purpose of this study, then, was to outline the physiological ontogeny of the cardio-respiratory system of the developing blue gourami ($Trichogaster\ trichopterus$), and to determine developmental stages and physiological states associated with the transition from aquatic to bimodal respiration.

3.2. Materials and Methods

Larvae were reared as described in Chapter 1 (Section 1.2.2: Care and Maintenance of Larvae). Food was withheld from experimental animals for 18 to 24 h prior to making any physiological measurements. Abbreviations of physiological parameters measured are provided in Table 3.1.
3.2.1. Heart Rate

Heart rate ($f_{H}$; beats · min$^{-1}$) was recorded from 1-15 dpf by direct observation via light microscopy. Beyond this age, however, diminishing body transparency no longer allowed for visual observation of the heart. Larvae were placed in holding chambers within a Petri dish filled with air-saturated water (~50 ml). This dish was placed on a glass dish through which water at $27^{\pm}0.5^\circ$C circulated to maintain appropriate temperature. The entire apparatus was then placed on the stage of a stereomicroscope. Larvae were held for 5 min prior to taking measurements, which was determined to be a sufficient interval for $f_{H}$ to stabilize, but not so long that declining oxygen within the chamber would become a factor. Larval heart rate was determined by averaging three 15 s counts.

3.2.2. Gill Ventilation Rate

Gill ventilation rate ($f_{GV}$; opercular beats · min$^{-1}$) was determined through microscopic examination in the same manner as described above for $f_{H}$ for larval gouramis (1-15 dpf).

3.2.3. Hypoxia-Stimulated Onset of Air-Breathing

To determine the point in development that air breathing becomes possible, larvae were exposed to extreme aquatic hypoxia to stimulate aerial respiration. Nitrogen gas was bubbled through 100 ml larval tank water ($27^{\pm}0.5^\circ$C) in an Erlenmeyer flask sealed with a rubber stopper for 1 h, resulting in 3-5% $O_2$ water, confirmed by routine testing with a microelectrode (see Section 3.2.6.). This level of
hypoxia was enough to cause loss of equilibrium after only a few minutes, and thus would be expected to elicit air breathing if the larva/juvenile was capable of this respiratory behavior. Nitrogen gas was also pumped into the air space above the water in order to remove the effects of aquatic surface respiration. Individual gouramis (5-60 dpf) were placed in the Erlenmeyer flask and were observed for signs of air breathing, as determined by a small air bubble expelled from the fish's opercula. If no air breathing was observed before loss of equilibrium, the larva was respiring completely aquatically, and it was presumed that air breathing was not yet possible for that animal. If air breathing was observed, the gourami was considered to have transitioned to bimodal respiration.

3.2.4. Air Breathing Frequency in Normoxia

Air breathing frequency (f_{AB}) in gourami larvae was also monitored in normoxia (5-60 dpf). Individual gouramis were introduced into 250 ml glass containers with normoxic water flowing through at 50 ml \cdot \text{min}^{-1}. This rate enabled complete water turnover of the chamber approximately every 5 min, which ensured maintenance of normoxia, as determined via testing with a microelectrode (see Section 3.2.6.). Containers were maintained at 27\pm0.5^\circ\text{C}. Animals were held for 0.5 h prior to measurements. Digital video recordings were made for 20 min, with three trials for each animal. The recordings were analyzed to determine if air breathing was taking place, and if so, at what rate.
3.2.6 Aquatic/Aerial Oxygen Consumption

Closed respirometry, based on a method described by Barrionuevo and Burggren (1999), was performed with embryos/larvae from 1 to 20 dpf. Briefly, embryos/larvae were placed in 3 or 5 ml glass syringes (depending on larval size) filled with O₂-saturated tank water. Water was injected from the syringes into a flow-through O₂ microelectrode (Model 16-730, Microelectrodes, Inc., NH) inserted in an aluminum block, through which 27±0.5°C water was circulated to ensure a constant sample temperature for each reading. The voltage output from the microelectrode was converted into partial pressure via Chart software (Power Lab, Chart 5, ADInstruments) to obtain the initial PO₂ of the water in the syringes. The syringes were then held in a 27±0.5°C water bath for 1 h, after which water samples from the syringes were again injected into the microelectrode to obtain a second PO₂ reading. Total oxygen consumption (\(\dot{V}O_2\)) was calculated using Equation 3.1, and mass-specific \(\dot{V}O_2\) (\(\dot{M}O_2\)) was determined by dividing this value by total body wet mass (mg).

Equation 3.1. \[ \dot{V}O_2 = \frac{\mu \text{mol O}_2 \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}}{\Delta t} = \frac{\Delta P O_2 \cdot \alpha \cdot V}{\Delta t} \]

where \(\Delta P O_2\) is the change in PO₂ over recorded interval (torr); \(\alpha\) is the solubility coefficient of O₂ at 27°C (1.617 \(\mu\)mol \cdot l⁻¹ \cdot torr⁻¹), \(V\) is the volume of water in respirometer (l), and \(\Delta t\) is the time elapsed between O₂ readings (h).

Both aquatic and aerial respirometry studies were conducted on gouramis 21 dpf and older using a variation of the syringe respirometry technique mentioned previously. Older larvae and juveniles were introduced into 5 or 10 ml glass syringes filled with air-saturated tank water. Depending on the size of the syringe used, 0.5 ml or
1.0 ml of air was then sucked into the top of the syringe. The conical shape at the top of the syringe allowed for a reduced air-water interface, and the syringe was held still and in an upright position until after measurements were taken to further keep these two media distinct. After 1 h in a 27±0.5°C water bath, a fine piece of polyethylene tubing was attached to the needle on the syringe, which was connected to the flow-through micro-electrode. With the syringe still upright, the gas at the top of the syringe was injected through the electrode to determine the PO$_2$ of the gaseous phase in the respirometer. Aerial oxygen uptake could then be determined from Equation 3.2.

\begin{equation}
\dot{V}O_2=(\mu\text{mol O}_2 \cdot \text{mg}^{-1} \cdot \text{hr}^{-1}) = \frac{\Delta P O_2 \cdot V}{\Delta t}
\end{equation}

where $\Delta P O_2$ is the change in PO$_2$ over recorded interval (torr); $V$ is the volume of air in respirometer (l), and $\Delta t$ is the time elapsed between O$_2$ readings (h).

Following measurement of the gas phase, water samples were injected to obtain the aquatic readings, as described previously. Pilot studies confirmed an adequate separation of the aquatic and aerial phases. Strictly aquatic larvae (<20 dpf) were placed in a syringe with only water or with water and an aerial phase. After 1 h, a similar significant reduction in aquatic PO$_2$ was observed for both syringes, while aerial PO$_2$ remained unchanged in the syringe containing air.
3.2.7. Statistical Analyses

Data were analyzed for statistically significant differences throughout development using One Way ANOVA with post-hoc tests, or Kruskal-Wallis One Way ANOVA on Ranks with appropriate post-hoc tests (e.g. Holm-Sidak Multiple Comparisons Procedure), if non-parametric alternatives were required. Values given are Mean ± 1 SE. Regression analysis was employed when data were analyzed as a function of body mass. All statistical tests adopted a significance level of p=0.05, and were conducted with SigmaStat (Systat) software.

3.3. Results

3.3.1. Heart Rate

Heart rate of blue gourami larvae was observed from 1-15 dpf, and data were grouped into “bins” of 2 dpf (Figure 3.1). Heart rate increased significantly (p<0.001) during development, with notable increases between the embryonic state at 1 dpf (~125 beats · min⁻¹) and the early larval stages at 3-5 dpf (~260 beats · min⁻¹). Subsequently, $f_h$ declined slightly, but remained relatively stable at ~230 beats · min⁻¹ thereafter.

3.3.2. Gill Ventilation Rate

Gill ventilation rate was examined from 1-15 dpf, with data grouped into “bins” of 2 dpf. An overall significant (p<0.001) increase in $f_{GV}$ was observed for this developmental period (Figure 3.2). No opercular movements were observed in embryos or larvae before 3 dpf (0 beats min⁻¹), but by 3-5 dpf, $f_{GV}$ had increased to ~50
beats · min⁻¹. Gill ventilation rate peaked at 7 dpf (~160 beats · min⁻¹), and remained relatively stable at ~130 beats · min⁻¹.

3.3.3. Hypoxia-Stimulated Onset of Air Breathing

Gourami larvae acutely exposed to extreme hypoxia (~3-5% O₂) were monitored for signs of air breathing from 5 to 60 dpf, with larvae grouped into “bins” of 5 dpf (Figure 3.3). No air breaths were observed prior to 20 dpf (0 breaths h⁻¹), but air breathing attempts were present by 25 dpf (21±14 breaths · h⁻¹) and for the remainder of the time observed. Thus, the onset of air breathing for gouramis reared in normoxia is between 20 and 25 dpf.

3.3.4. Air breathing Frequency in Normoxia

Air breathing frequency (f_{AB}) of blue gourami reared in normoxia was observed from 5-60 dpf, with data grouped into “bins” of 5 dpf. Air breathing frequency increased significantly (p<0.001) over the time interval studied (Figure 3.4). As with larvae exposed to extreme hypoxia, no successful air breathing attempts were observed in normoxia prior to 20 dpf, but f_{AB} increased to ~3 breaths · h⁻¹ by 25 dpf, peaked at 30 dpf (~30 breaths · h⁻¹), and then leveled off at ~15 breaths · h⁻¹ by 40-60 dpf.

3.3.5. Oxygen Consumption

Aquatic and aerial oxygen consumption rates (V̇O₂; µmol O₂ · h⁻¹) were recorded from 1-60 dpf, with data grouped into “bins” of 5 dpf. Total oxygen consumption increased significantly (p<0.001), from ~0.01 µmol O₂ · h⁻¹ at 1 dpf to ~0.87 µmol O₂ ·
h\(^{-1}\) at 60 dpf (Figure 3.5-A). Although there were no significant differences between any adjacent grouped dpf, there were significant increases in \(\dot{V}O_2\) between 5 dpf and 20 dpf, and between 15 dpf and 30 dpf. Aquatic \(\dot{V}O_2\) showed a similar significant (p<0.001) increase (Figure 3.5-A), from \(\sim 0.01 \mu\text{mol O}_2 \cdot h^{-1}\) at 1 dpf to \(\sim 0.65 \mu\text{mol O}_2 \cdot h^{-1}\) by 60 dpf. Aerial \(\dot{V}O_2\) also significantly (p<0.001) increased (Figure 3.5-A), from 0 \(\mu\text{mol O}_2 \cdot h^{-1}\) at 0-20 dpf, to \(\sim 0.22 \mu\text{mol O}_2 \cdot h^{-1}\) by 60 dpf. Significant increases occurred between 20 dpf and 40 dpf. There was a significant (p<0.001) positive relationship of \(\dot{V}O_2\) as a function of body mass for blue gouramis in this study, with a scaling exponent of 0.77.

Mass-specific oxygen consumption rates (\(M_O_2; \mu\text{mol O}_2 \cdot mg^{-1} \cdot h^{-1}\)) differed significantly (p<0.001) over the time period observed (Figure 3.6-A), though the difference was not consistent. For example, \(M_O_2\) increased slightly between the embryonic stage (1 dpf, \(\sim 0.10 \mu\text{mol O}_2 \cdot mg^{-1} \cdot h^{-1}\)) and early larval stages (5-10 dpf, \(\sim 0.13 \mu\text{mol O}_2 \cdot mg^{-1} \cdot h^{-1}\)), before declining significantly by 30 dpf (\(\sim 0.04 \mu\text{mol O}_2 \cdot mg^{-1} \cdot h^{-1}\)), where it remained rather stable through 60 dpf. Aquatic \(M_O_2\) showed a similar significant (p<0.001) difference over the time period studied (Figure 3.6-A). Again, the trend was a slight increase between 1 dpf and 5 dpf, followed by a significant decline through 30 dpf (\(\sim 0.035 \mu\text{mol O}_2 \cdot mg^{-1} \cdot h^{-1}\)), with no further significant changes through 60 dpf. Aerial \(M_O_2\) was opposite of either total or aquatic values, as this showed a significant (p<0.001) increase over the time period observed (Figure 3.6-A), from 0 \(\mu\text{mol O}_2 \cdot mg^{-1} \cdot h^{-1}\) (1-20 dpf) to \(\sim 0.007 \mu\text{mol O}_2 \cdot mg^{-1} \cdot h^{-1}\) (40 dpf). No further significant changes were observed through 60 dpf. There was a significant (p<0.001) negative relationship of \(M_O_2\) as a function of body mass for blue gouramis in this study (Figure 3.6-B), with a scaling exponent of -0.23.
3.4. Discussion

3.4.1. Heart Rate

The pattern of heart rate observed for gouramis in early development (Figure 3.1) is similar to that of a number of other developing Teleost fishes (e.g. Holeton, 1971; McDonald and McMahon, 1977; Barrionuevo and Burggren, 1999; Bagatto, 2005). For example, $f_H$ of embryonic zebrafish is $\sim$125 beats $\cdot$ min$^{-1}$ (28°C), but then increases to 175 beats $\cdot$ min$^{-1}$ by 10-30 dpf, before declining to $\sim$130 beats $\cdot$ min$^{-1}$ by 100 dpf (Barrionuevo and Burggren, 1999). This similarity may be indicative of shared patterns of cardiac development and regulation among Teleost fishes, and perhaps among all vertebrates, as cardiac and respiratory systems mature. In fact, the most radical changes in the physiology of the zebrafish cardiovascular system coincide with the maturation of the heart to its adult form, and with the transition from diffusive to conductive oxygen supply within the animal (Pelster and Burggren, 1996). Although convective transport is not required in the zebrafish until 14 dpf (Jacob et al., 2002), the first steps towards this transition may occur earlier in development. In 5-6 dpf zebrafish larvae, for example, there is an increased red blood cell velocity and cardiac output, and decreased cross-sectional area of key vessels (Bagatto and Burggren, 2006). It is at about this same time period that $f_H$ peaks (Figure 3.1) and that onset of gill ventilation (Figure 3.2) occurs in blue gouramis. Thus, developing blue gouramis may also start the transition to conductive transport during this same time period.

While no studies could be found that observed $f_H$ of developing air breathing fishes, rates are similar among many adult air breathing fishes. For example, $f_H$ of the adult climbing perch (Anabas testudineus), an Anabantid, is 25 to 62 beats $\cdot$ min$^{-1}$ (pre-
and post-air breath, respectively; 25°C) (Singh and Hughes, 1973), while that of the adult air breathing catfish *Clarias batrachus* is 30 to 39 beats · min⁻¹ (pre- and post-air breath, respectively; 25°C) (Jordan, 1976). Similarly, $f_H$ of the facultative air breathing jeju (*Hoplerythrinus unitaeniatus*) (Oliveira et al., 2004) and swamp eel, *Synbranchus marmoratus* (Skals et al., 2006) were ~40 beats · min⁻¹ (25°C).

The relatively high $f_H$ of blue gouramis through 15 dpf closely mirrors the elevated $\dot{MO}_2$ during this period (see Section 3.4.5: Oxygen Consumption). However, the decreasing trend of both $f_H$ (through 15 dpf) and $\dot{MO}_2$ (through 60 dpf) may be directly associated with increasing mass during development, and it is quite feasible that $f_H$ of adult gouramis is similar to the other air breathing fishes mentioned. Although ventilation tachycardia (i.e. increased $f_H$ following an air breath) occurs in a number of adult air breathing fishes (Singh and Hughes, 1973; Burggren and Pinder, 1991; Graham et al., 1995; Skals et al., 2006), this is likely not a factor when considering the data presented here, as air breathing in blue gourami larvae has not begun by 15 dpf.

3.4.2. Gill Ventilation Rate

The onset of gill ventilation in zebrafish appears to follow a similar pattern as that reported here for blue gouramis (Figure 3.2), with $f_{GV}$ initially low and irregular, but increasing to ~45 beats · min⁻¹ by 7-9 dpf (28°C). However, $f_{GV}$ declines by 10 dpf to only a few beats · min⁻¹ (Jonz and Nurse, 2005). The duration of this reduced $f_{GV}$ during development is unknown, nor is the cause, for $f_{GV}$ of adult zebrafish again increases to ~160 beats · min⁻¹.
Blue gourami larvae maintain a relatively high \( f_{GV} \) from 7 dpf through at least 15 dpf (Figure 3.2). If developing gouramis follow a similar pattern of gill ventilation as zebrafish, increased \( f_{GV} \) at these stages may reflect ionoregulatory, rather than purely respiratory functions (Rombough, 2002, 2007). Rombough (2004) estimated that, based on the relative cutaneous surface area (i.e. \( SA_C \) per unit oxygen uptake and body mass), cutaneous oxygen uptake becomes limiting for zebrafish at \(~0.3\) mg, or at \(~10-14\) dpf. It is at about this time that branchial respiration becomes necessary. If the parameters for this estimation are similar for blue gouramis, cutaneous oxygen uptake should become limiting for this species at 7-10 dpf, but further studies are necessary to confirm this.

The gill ventilation rate of blue gouramis at 7-9 dpf is more than doubled over that of zebrafish of the same age. As ventilatory movements are relatively costly (i.e. averaging \(~10\%\) of total oxygen uptake) (Hughes, 1973; Farrell and Steffensen, 1987; Graham et al., 1987), maintaining a high \( f_{GV} \) would not be practical if this cost was not offset by some benefit. The rapid \( f_{GV} \) during this time may also be an early indication of the reduced relative growth of gills in the blue gourami. As discussed in Chapter 2, adult air breathing fishes generally show reduced gill surface area. For the blue gourami and other Anabantid fishes, this is mostly a result of the third and fourth gill arches being reduced to serve as blood shunts to prevent branchial oxygen loss to hypoxic waters (Burggren, 1979; Prasad, 1988; Munshi and Hughes, 1992; Huang et al., 2007). The relative slowing of the growth and proliferation of the filaments and secondary lamellae in this species may begin as early as the larval stages.
As teleost development progresses, the contribution of cutaneous respiration decreases, while that of branchial respiration increases (see Rombough, 2007). Additionally, the increasing blood-water diffusion distance of the secondary lamellae noted for Anabantids during late larval stages (Mishra and Singh, 1979; Prasad, 1989) may explain the need for a continued high gill ventilation rate in larval blue gouramis prior to the onset of air breathing. Following the transition to bimodal respiration, aerial oxygen uptake via the labyrinth organ can supplement aquatic respiration, allowing $f_{GV}$ to decline, especially immediately following an air breath (e.g. Graham, 1983; Ar and Zacks, 1989). For example, pre-air breath $f_{GV}$ of the adult air breathing catfish *Clarias lazera* is 44 beats · min⁻¹, but declines to 24 beats · min⁻¹ post-air breath (Ar and Zacks, 1989). However, air breathing has not commenced in the blue gourami by 15 dpf, and thus fluctuations of $f_{GV}$ associated with air breathing were not a factor in this study.

3.4.3. Hypoxia-Stimulated Onset of Air Breathing

Larval exposure to extreme hypoxia results in loss of equilibrium and ultimately death for zebrafish larvae (Ho, 2008) and adults (Rees et al., 2001). This formed the basis for the rationale that, once air breathing was morphologically and physiologically possible for developing gourami larvae, air breathing attempts would certainly be made in extreme hypoxia, as the animal would be incapable of acquiring adequate oxygen from the water. Thus the earliest onset of air breathing would be observable. While no air breathing attempts occurred prior to 20 dpf (Figure 3.3), larvae spent the majority of time at or near the surface, presumably attempting aquatic surface respiration. The first successful attempts at air breathing occurred by 25 dpf, but these events were erratic
and often appeared to be the result of “accidental” uptake of gas as the fish frantically swam about at the water’s surface. Air breathing frequency increased thereafter, with air breathing attempts becoming more regular and well coordinated throughout development.

The onset of air breathing at 25 dpf coincides with larvae first attaining $L_{TB}$ of 10-12 mm, which is the point in development when most other Anabantids begin air breathing (Das, 1927; Mishra and Singh, 1979; Prasad and Singh, 1984). As exposure to extreme hypoxia (Figure 3.3) did not cause air breathing to commence earlier in development than normoxia (Figure 3.4), it is possible that the onset of air breathing is limited by a factor other than the need to breathe air. Indeed, some aquatic air breathing fishes begin surfacing prior to the complete development of their air breathing organ (Bader, 1937; Prasad and Prasad, 1985; Munshi and Hughes, 1986). Perhaps a critical size must be attained to allow the animal to overcome the water-surface tension, or to deal with the added buoyancy resulting from the inspired air (Graham, 1997).

3.4.4. Air Breathing Frequency in Normoxia

Blue gouramis exposed to normoxic water also began air breathing by 25 dpf (Figure 3.4), but at about one-tenth the rate of those in extreme hypoxia. This is likely an indication that air breathing is mostly facultative at this point in development. As mentioned, some gourami larvae first attain a total body length of 10-12 mm by 25-30 dpf, which is when most Anabants begin air breathing. Additionally, some larvae reach a body mass $>12$ mg at this time, which is when the rate of increasing $L_{TB}$ was found to slow with further increases in body mass (see Figure 2.6). Mishra and Singh
(1979) similarly reported that growth of larval *A. testudineus* immediately prior to the onset of air breathing owes mostly to rapid increases in girth, rather than length.

The sudden increase in $f_{AB}$ between 25 dpf and 30 dpf is partially due to a larger proportion of the observed larvae air breathing (i.e. some of the larvae at 25 dpf were not air breathing, thus mean $f_{AB}$ was reduced on that day), as well as to an overall increase in $f_{AB}$. The elevated $f_{AB}$ at 30-35 dpf may represent fishes habituating to this new lifestyle, or may be that smaller fish have different respiratory efficiencies or air convection requirements than their larger, older cohorts (Jordan, 1976; Burggren, 1979, Graham, 1997). Air breathing frequency is high at the onset of bimodality for *Colisa fasciatus*, but then slows considerably as the animal matures (Prasad and Singh, 1984). Likewise, for the developing gourami, $f_{AB}$ after 35 dpf began to decline until reaching ~12 breaths·h$^{-1}$ at 60 dpf, similar to the $f_{AB}$ for adults of this species (Burggren, 1979).

3.4.5. Oxygen Consumption

All oxygen uptake was via aquatic respiration prior to the onset of air breathing at 25 dpf, after which time the relative contribution of aquatic respiration declined, while that of aerial respiration increased. At 25 dpf aquatic $\dot{M}O_2$ accounted for ~97% of total $\dot{M}O_2$, while aerial $\dot{M}O_2$ only contributed ~3%. By 40-60 dpf, aquatic $\dot{V}O_2$ was responsible for ~78% of total $\dot{V}O_2$, while the aerial contribution rose to ~22%.

Juvenile *Colisa fasciata* obtain ~36% of total $\dot{V}O_2$ via aerial respiration (Prasad and Singh, 1984), and for juvenile *A. testudineus*, the aerial contribution is ~40% (Mishra and Singh, 1979). Burggren (1979) similarly calculated that ~60% of $\dot{V}O_2$ for adult blue gouramis is via aquatic respiration, while aerial respiration via the labyrinth
organ accounts for ~40%. While the average aerial contribution for juvenile (30-60 dpf) blue gouramis was lower than that of adult gouramis and juveniles of other species, there were individuals for which ~40% of total \( V_{O_2} \) was via aerial respiration. Conversely, there were individual animals for which only 5-10% of total \( V_{O_2} \) was obtained by air breathing. These animals had a similar body length and mass as their cohorts, and so it is unclear why some would reveal particularly elevated or reduced rates of aerial oxygen uptake.

The mass-specific oxygen consumption rate generally declined throughout development, as would be expected with increasing body mass (Brown and West, 2000; Burggren, 2005). An exception to this occurred between 1 dpf and 5 dpf, at about the time of hatching, when \( MO_2 \) increased from ~0.09 to ~0.14 \( \mu \text{mol } O_2 \cdot \text{mg}^{-1} \cdot \text{h}^{-1} \). Developing zebrafish show a similar but more extreme early rise in \( MO_2 \), with a 10-fold increase between hatching (0.004 \( \mu \text{mol } O_2 \cdot \text{mg}^{-1} \cdot \text{h}^{-1} \)) and 10 dpf (0.04 \( \mu \text{mol } O_2 \cdot \text{mg}^{-1} \cdot \text{h}^{-1} \)), before decreasing to ~0.005 \( \mu \text{mol } O_2 \cdot \text{mg}^{-1} \cdot \text{h}^{-1} \) by 60 dpf (Barrionuevo and Burggren, 1999). One explanation for the initial increase in \( MO_2 \) is that organogenesis and the conversion of yolk to biomass are taking place at this time (Barrionuevo and Burggren, 1999). Another possibility is that, at hatching, removal of the chorion and perivitelline fluid surrounding the embryo decreases the oxygen diffusion barrier, and thus the newly hatched larva is no longer limited to the same extent as it was as an embryo (Gruber and Wieser, 1983; Rombough, 1988; Warkentin, 2007). Mass-specific oxygen consumption rate of the developing Anabantids \textit{Colisa fasciatus} (12 mg; 0.038 \( \mu \text{mol } O_2 \cdot \text{mg}^{-1} \cdot \text{h}^{-1} \)) and \textit{Anabas testudineus} (15 mg; 0.043 \( \mu \text{mol } O_2 \cdot \text{mg}^{-1} \cdot \text{h}^{-1} \)) (Prasad, 1989) are similar to the values reported here for developing gouramis of about the same
size (12 mg; ~0.03 µmol O$_2$·mg$^{-1}$·h$^{-1}$), and thus have similar metabolic demands throughout development, as would be expected for such closely related species.

3.5. Summary

While much is known about the physiology of adult air breathing fishes, few studies have investigated the physiological ontogeny of these fishes. The purpose of this study was to observe cardio-respiratory development of an air breathing Anabantid fish, the blue gourami, reared in normoxia through 60 dpf. This developmental period includes a completely aquatic phase and subsequent transition to bimodal breathing.

The heart rate of developing blue gouramis follows a similar pattern as many other teleost fishes, with a sharp increase around the time of hatching followed by a steady decline through 15 dpf. Gill ventilation was not observed until 3 dpf, when opercular movements were slow and appeared uncoordinated. By 7 dpf the rate of gill ventilation had increased significantly, and remained elevated through 15 dpf. Air breathing in this species commenced by 25 dpf. Exposure to extreme hypoxia did not affect the timing of the onset of air breathing compared to normoxic exposure, but did increase the rate of air breathing. Total oxygen consumption rates increased throughout development, with the aerial contribution increasing, and the aquatic contribution decreasing, after 25 dpf. Total and aquatic mass-specific oxygen consumption rates sharply increased early in development, but then declined with further growth. However, aerial mass-specific oxygen consumption increased between 25-60 dpf, suggesting the importance of aerial respiration for this species as development progresses.
Table 3.1. Abbreviations of physiological parameters studied.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Physiological Parameter</th>
<th>Units</th>
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<tbody>
<tr>
<td>f_{AB}</td>
<td>Air Breathing Frequency</td>
<td>breaths · hour^{-1}</td>
</tr>
<tr>
<td>f_{GV}</td>
<td>Gill Ventilation Rate</td>
<td>(opercular) beats · min^{-1}</td>
</tr>
<tr>
<td>f_{H}</td>
<td>Heart Rate</td>
<td>(heart) beats · min^{-1}</td>
</tr>
<tr>
<td>V_{O_2}</td>
<td>Oxygen consumption rate</td>
<td>µmol O_2 · h^{-1}</td>
</tr>
<tr>
<td>M_{O_2}</td>
<td>Mass-Specific V_{O_2}</td>
<td>µmol O_2 · mg · h^{-1}</td>
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</tbody>
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Figure 3.1. Heart rate ($f_{H}$) as a function of development of blue gourami larvae reared in normoxia through 15 dpf. n values given for each grouped dpf. Letters indicate grouped dpf that are not statistically different. Overall significance: p<0.001.
Figure 3.2. Gill ventilation rate ($f_{GV}$) as function of development of blue gourami larvae reared in normoxia through 15 dpf. n values given for each grouped dpf. Letters indicate grouped dpf that are not significantly different. Overall significance throughout development: p<0.001.
Figure 3.3. Onset of air breathing as stimulated by extreme hypoxia (3-5% $O_2$; aquatic and aerial) as function of development of blue gouramis reared in normoxia. Data analyzed for significance in Chapter 5.
Figure 3.4. Air breathing frequency ($f_{AB}$) measured in normoxia (aquatic and aerial) as function of development of blue gouramis reared in normoxia through 60 dpf. n values given for each grouped dpf. Letters indicate grouped dpf that are not significantly different. Overall significance throughout development: $p<0.001$. 
Figure 3.5. (A) Total oxygen consumption rate (\(\dot{V}O_2\)) as function of development for gouramis reared in normoxia through 60 dpf. n values for Total are same for Aquatic and Aerial. Letters indicate grouped dpf that are not significantly different. Overall significance throughout development: p<0.001 for all data sets. (B) \(\dot{V}O_2\) as function of total body wet mass. Regression line with 95% confidence intervals and equation given.
Figure 3.6. (A) Mass-specific oxygen consumption rates ($\dot{M}O_2$) as function of development of gouramis reared in normoxia through 60 dpf. n values for Total same as for Aquatic and Aerial. Letters indicate grouped dpf not significantly different (a-b: Total/Aquatic; α-β: Aerial). Overall significance throughout development: $p<0.001$ for all data sets. (B) $M_O_2$ as function of total body wet mass. Regression line with 95% confidence intervals and equation given.
CHAPTER 4
HYPOXIA-INDUCED DEVELOPMENTAL PLASTICITY
OF RESPIRATORY STRUCTURES OF THE BLUE GOURAMI

4.1. Introduction

4.1.1. Environmental Influences and Developmental Plasticity

Survival requires that environmental disturbances be met by an animal’s ability to cope with external changes. Whereas adult animals generally must modify established physiological processes to survive environmental perturbations, embryonic or larval animals still growing and differentiating may be capable of showing developmental plasticity—that is, altered rates of development and/or modified phenotypes resulting from variant environments may allow exposed animals to become more adept at dealing with similar, and even more extreme, environmental conditions (West-Eberhard, 1989; Moore et al., 2006). A number of environmental factors can evoke developmental plasticity, including oxygen availability. Hypoxia is a common occurrence in many freshwater habitats, and animals living in such habitats have evolved to deal with this stress. However, as selection pressures exist for not only adults, but for every life history stage, it is important to understand how environmental factors shape developing animals. Thus, the purpose of this study is to observe morphological plasticity of an air breathing fish, the blue gourami (Trichogaster trichopterus), in response to rearing in chronic hypoxia, with particular emphasis on development of respiratory structures.
4.1.2. Hypoxia and the Evolution of Air Breathing

Not all aquatic environments are always air-saturated. Due to water’s properties, its oxygen content is not only ~1/40\textsuperscript{th} that of air, but is also more highly variable (Johansen, 1970; Graham, 1997; Schmidt-Nielsen, 1997). Although surface water is in equilibrium with air, sub-surface water must be oxygenated via photosynthesis or stirring. Many aquatic environments routinely experience hypoxia, especially those with little photosynthesis, deficient or nonexistent flow or vertical mixing, or an excess of organismic respiration and organic decomposition (Johansen, 1970; Munshi and Hughes, 1992; Timmerman and Chapman, 2004). Depending on biotic and abiotic factors, aquatic environments can experience chronic, seasonal, or diel hypoxia (Dehadrai and Tripathi, 1976; Graham, 1997; Chapman \textit{et al.}, 2002).

Under such unfavorable conditions as hypoxia presents, complex animals with relatively high metabolic rates such as fishes must either move to more favorable microhabitats or cope with the effects of hypoxia. Adaptations of completely aquatic fishes to hypoxia include increased surface area or perfusion of the gills (McDonald and McMahon, 1977; Booth, 1979; Chapman \textit{et al.}, 2000; Sollid \textit{et al.}, 2003; Sollid and Nilssen, 2006), increased ventilation frequency and/or amplitude (Randall, 1970; Hochachka, 1980; Jonz and Nurse, 2005), marked bradycardia and increased blood pressure (Randall and Shelton, 1963; Holeton and Randall, 1967; Fritsche and Nilsson, 1989; Farrell, 2007), and aquatic surface respiration (Lewis, 1970; Kramer, 1983; Gee and Gee, 1995).

Other aquatic vertebrates, including amphibians and some fishes, are capable of escaping the effects of hypoxic stress by becoming reliant upon atmospheric, rather
than aquatic oxygen, via “air gulping” (e.g. Burggren, 1982, Gee and Gee, 1995) and various forms of air breathing (see Chapter 1: Introduction). A variety of organs have evolved in fishes that allow for aerial oxygen uptake (see Graham, 1997 for review). While severe hypoxic exposure can retard overall development or even be lethal to developing animals, milder hypoxia may result in accelerated or augmented organ and organ system development. Developmental plasticity of organs and systems related to oxygen acquisition and transport may be especially prevalent when animals are reared in hypoxia. Adult fishes can also modify respiratory structures in response to changes in environmental conditions, but this seems to be an exception rather than the rule (Sollid et al., 2003; Sollid and Nilsson, 2006, 2007).

4.1.3. Morphological Responses of Teleost Fishes to Hypoxia

The effects of hypoxic exposure upon developing vertebrate morphology are inconsistent. This is likely 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, rearing Atlantic cod (Chabot and Dutil, 1999) and turbot (Pichavant et al., 2000) in 10% O$_2$ resulted in significantly decreased growth rates, while no effects were observed in Atlantic menhaden and spot at hypoxic rearing levels above ~5% O$_2$ (McNatt and Rice, 2004). African cichlids developing in hypoxic (~3% O$_2$) waters 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), while Arctic char reared in hypoxia (4-5% O$_2$) for 47 d
post-hatch showed no significant difference in overall gill surface area (McDonald and McMahon, 1977).

No study to date could be found that observed the effects of rearing an air breathing fish in hypoxia. Thus, the goal of the present study is to determine the extent to which morphological parameters are altered in blue gouramis in response to chronic moderate hypoxia. Especially of interest are measurements of the whole body (length, mass, surface area) and the respiratory structures (gills and labyrinth organ).

4.2. Materials and Methods

4.2.1. Establishment of Hypoxic Conditions

Similar to the experiments described in Chapters 1–3, embryos were removed from the bubble nest within 12 h of fertilization and transferred to one of two 18 L glass tanks filled with ~3 L tank water. These tanks were labeled either normoxic/control (Norm) or hypoxic/experimental (Hyp). Both tanks were placed into larger (38 L) sealed glass tanks to maintain high humidity. Atmospheric air was bubbled through the water of the Norm tank to maintain normoxia (20.9% O$_2$). For the Hyp tank, nitrogen was introduced into the space above the water, regulated by an oxygen controller (ProOx, BioSpherix) to achieve a constant level of aerial hypoxia (13.0±0.1% O$_2$). An air pump was then placed in the larger tank to bubble the 13% O$_2$ gas through the water to attain an equivalent level of aquatic hypoxia. The oxygen controller was calibrated weekly with nitrogen gas (0% O$_2$) and air (20.9% O$_2$). The oxygen level of the water of each rearing group was confirmed several times each week via the microelectrode/PowerLab setup described in Chapter 3.
All other aspects of larval care were similar between groups, as described in Chapter 1 (Section 1.2.2. Care and Maintenance of Larvae).

A level of hypoxia of 13% was chosen for a number of reasons. Pilot studies using 10% O$_2$ produced gourami larvae with significant differences in resting heart rate compared to normoxic controls when both groups were measured in air-saturated (normoxic) water, which was a desired physiological response. However, this level of hypoxia also resulted in gross morphological deformities (Figure 4.1), and larvae did not survive past 8 dpf. As larvae were introduced prior to 24 h post-fertilization, these deformities could possibly have been avoided by postponing the initial exposure, and thus potentially avoiding a “critical window” of development. In fact, a second pilot study confirmed that larvae can indeed survive 10% O$_2$ when introduced after 3 dpf. However, I wanted to expose all developmental stages to a constant hypoxic environment in order to observe as many developmental responses as possible. A third study exposed embryos to 15% O$_2$ and found that heart rate of these larvae measured in normoxic water was not significantly different than the control group, suggesting that this “dose” of hypoxia was too mild to produce desired physiological effects. Additionally, oxygen consumption studies found that Norm larvae were able to maintain a stable $\dot{V}O_2$ below 10% O$_2$. Thus, an intermediate level of hypoxia (between 10% and 15% O$_2$) was chosen as the experimental level, as this would likely produce morphological and physiological differences between groups, but that these differences would not have fatal outcomes.
4.2.2. Measurements of Morphological Development

Food was withheld from experimental larvae for 18 to 24 h prior to making any morphological measurements. All measurements of whole body (total body length; total body wet mass; cutaneous surface area), gill structures and labyrinth organs for both Norm and Hyp were made as described in Chapter 2.

4.2.4. Statistical Analyses

Data were analyzed for significant differences between Norm and Hyp, and throughout development within each group using Two-Way ANOVA or a non-parametric alternative, if required. If a significant interaction between Rearing Group and Development (dpf) was found, a post-hoc test (e.g. Holm-Sidak Multiple Comparisons Procedure) was employed. When data were evaluated as a function of body mass, regression analyses were used, and t-tests were utilized to compare the slopes of regression lines between groups. P values reported for tests between groups represent the significance between rearing groups (Norm vs. Hyp). Values of respiratory surface area by structure that were analyzed as a percent of total respiratory surface area were first arcsin transformed before performing appropriate statistical tests. All statistical tests adopted a significance level of p=0.05, and were analyzed with SigmaStat (Systat) software. All statistics of morphological development are summarized in Tables 4.1-4.4.
4.3. Results

4.3.1. Whole Body Size and Shape

Total body length (mm) of the Hypoxic group (Hyp) as a function of development significantly (p<0.001) increased in a linear fashion over the observed time period, from ~3 mm up to ~20 mm by 90 dpf (Figure 4.2-A). When body length of Hyp was compared to that of the Normoxic Group (Norm), there was no significant difference between groups over the developmental period studied.

Total body wet mass (mg) of Hyp as a function of development significantly (p<0.001) increased from 2 up to 90 dpf (Figure 4.2-B). Body mass increased from ~0.1 mg to ~130 mg over the time period observed, and was best described by a quadratic function. When body mass of Hyp was compared to that of Norm, no significant difference was determined.

Body length of Hyp as a function of body mass significantly (p<0.001) increased over the range of body mass observed (Figure 4.3). These data were best described by biphasic linear relationships, with regressions representing body length versus mass<12 mg and body length versus mass>12 mg. There was a significant (p<0.005) difference in the slope of body length between groups at mass<12 mg, with that of Hyp (b=0.55) less than Norm (b=0.71). No significant difference was observed between the two groups at mass>12 mg.

Cutaneous surface area (SA<sub>C</sub>; mm<sup>2</sup>) of Hyp as a function of development showed a significant (p=0.016) positive relationship (Figure 4.5-A). Similarly, SA<sub>C</sub> of Hyp as a function of body mass increased significantly (p<0.001) from ~38 to ~220 mm<sup>2</sup> over the range of body mass observed (Figure 4.5-B). The slopes of the regression lines
describing $SA_c$ as a function of body mass were not significantly different between Norm and Hyp.

In summary, with the exception of body length of larvae with mass $\leq$ 12 mg, rearing in chronic moderate hypoxia had no significant effects on the whole body morphology of blue gourami larvae or juveniles.

4.3.2. Development of Gills

The length of each gill arch of Hyp as a function of body mass showed a significant ($p<0.001$) positive relationship, for all arches (Figure 4.6-A). Gill arch length doubled, on average, over the developmental period observed. When gill arch length of Hyp was compared with that of Norm, the slope of Hyp at GA-IV ($b=0.0066$) was significantly ($p<0.05$) lower than that of Norm ($b=0.0084$), while that of Hyp at GA-VI ($b=0.0043$) was significantly ($p<0.025$) greater than that of Norm ($b=0.0026$). There was no significant difference between the slopes of either GA-III or GA-V, but the regression line for Hyp had a significantly lower elevation at GA-III ($p<0.01$), GA-IV ($p<0.05$), and GA-V ($p<0.01$).

The filament length at each gill arch of Hyp was similarly analyzed as a function of body mass, and a significant positive relationship was again observed at each gill arch (GA-III, GA-IV, GA-V: $p<0.001$; GA-VI: $p=0.002$). Filament length more than doubled on average over the range of body mass observed (Figure 4.6-B). When filament length of Hyp was compared with Norm, the slope of Hyp ($b=6.0 \times 10^{-3}$) was significantly ($p<0.025$) greater than that of Norm ($b=1.5 \times 10^{-3}$) at GA-VI, but no
significant difference in slope was observed at GA-III, GA-IV, or GA-V. However, the regression line for Hyp had a significantly (p<0.05) higher elevation than Norm at GA-IV.

The number of filaments at each gill arch (Figure 4.7-A) and the total number of filaments per animal (Figure 4.7-B) were analyzed for Hyp as a function of body mass. Filament count increased significantly (p<0.001) at all gill arches, nearly doubling on average over the developmental period observed. The total number of filaments per animal similarly showed a significant (p<0.001) increase, from 252 to 472 filaments. When the slopes of filament number of Hyp were compared with those of Norm, only that of GA-VI showed a significant (p<0.05) difference, with the slope of Hyp (b=0.07) greater than that of Norm (b=0.03).

There was a significant negative relationship of filament density of Hyp as a function of body mass (Figure 4.8) at GA-III (p=0.004) and GA-IV (p=0.006), but there was no relationship at GA-V or GA-VI. When the filament density at each gill arch of Hyp was compared to Norm data, there was no significant difference found between the slopes at any gill arch.

The number of lamellae per gill arch (Figure 4.9-A) and the total number of lamellae per animal (Figure 4.9-B) were analyzed as a function of body mass for Hyp. There was a significant (p<0.001) positive relationship found for number of lamellae at all gill arches, as well as for the total number of lamellae per animal. Total number of lamellae increased from ~2800 to ~11000 lamellae, representing ~4-fold increase over the range of body mass observed. When the number of lamellae per gill arch of Hyp was compared with Norm, no significant differences occurred between the slopes of the
regression lines at any gill arch. The total number of lamellae per animal similarly showed no significant difference between Hyp and Norm.

Lamellar density at each gill arch of Hyp was similarly analyzed as a function of body mass (Figure 4.10). A significant negative relationship was determined at GA-III (p=0.004) and GA-V (p=0.004), but no significance was found at GA-IV or GA-VI. When lamellar density at each gill arch of Hyp was compared to Norm data, there was no significant difference determined between the slopes at any gill arch.

There was a significant (p<0.001) positive relationship of single lamellar surface area (SA<sub>SL</sub>; mm<sup>2</sup>) as a function of body mass for Hyp (Figure 4.11-A) at GA-III, GA-IV, and GA-V, with SA<sub>SL</sub> more than doubling on average at these gill arches. No significance was noted at GA-VI. When SA<sub>SL</sub> of Hyp was compared to that of Norm, the slopes of Hyp at GA-III (b=3.86x10<sup>-5</sup>) and GA-V (b=1.90x10<sup>-5</sup>) were significantly (p<0.01) greater than those of Norm (GA-III: b=2.28x10<sup>-5</sup>, p<0.001; GA-V: b=1.23x10<sup>-5</sup>).

There was also a significant positive relationship of total lamellar surface area (SA<sub>L</sub>; mm<sup>2</sup>) as a function of body mass (Figure 4.11-B) for Hyp at all gill arches (GA-III, GA-IV, GA-V: p<0.001; GA-VI: p=0.015), with SA<sub>L</sub> increasing from 1.5-fold at GA-VI to more than 10-fold at GA-III. When SA<sub>L</sub> of Hyp was compared with that of Norm, the slope of Hyp (b=0.130) was significantly (p<0.001) greater than that of Norm (b=0.074) at GA-III, but no significant differences existed between the slopes at GA-IV, GA-V, or GA-VI.

Total lamellar surface area (SA<sub>TL</sub>; mm<sup>2</sup>) of Hyp as a function of body mass showed a significant (p<0.001) positive relationship, with SA<sub>TL</sub> increasing about 6-fold over the range of body mass observed, from ~20 mm<sup>2</sup> to ~120 mm<sup>2</sup> (Figure 4.12).
When $SA_{TL}$ of Hyp was compared with that of Norm, the slope of Hyp ($b=0.421$) was significantly ($p<0.001$) greater than that of Norm ($b=0.323$). When $SA_{TL}$ was considered as a mass-specific measurement ($\text{mm}^2 \cdot \text{mg}^{-1} \text{ body mass}$), Hyp was $\sim14\%$ greater than that of Norm.

4.3.3. Labyrinth Organ Development

Total labyrinth surface area ($SA_{Lb}; \text{mm}^2$) of Hyp as a function of body mass showed a significant ($p<0.001$) positive relationship, with $SA_{Lb}$ increasing from $\sim0.3 \text{ mm}^2$ to $\sim7 \text{ mm}^2$ (Figure 4.13). This represented more than a 20-fold increase over the range of body mass observed. $SA_{Lb}$ of Hyp was then compared with Norm, the slope of Hyp ($b=0.026$) was significantly ($p<0.025$) greater than that of Norm ($b=0.022$). When $SA_{Lb}$ was considered as a mass-specific measurement ($\text{mm}^2 \cdot \text{mg}^{-1} \text{ body mass}$), Hyp was $\sim36\%$ greater than that of Norm.

4.3.4. Total Respiratory Surface Area

Total respiratory surface area ($SA_{TR}; \text{mm}^2$) as a function of body mass showed a significant ($p<0.001$) positive relationship for Hyp, with values increasing from $\sim70 \text{ mm}^2$ to $\sim350 \text{ mm}^2$, more than 4-fold over the range of body mass studied (Figure 4.14-A). When $SA_{TR}$ of Hyp was compared to that of Norm, there was no significant difference between the slopes of the regression lines.

The growth of each respiratory structure (skin, gills, labyrinth) was also considered as a percent of $SA_{TR}$, taken as a function of body mass (Figure 4.14-B). The contribution of $SA_{C}$ to $SA_{TR}$ did not change for Norm ($\sim75\%$), but declined
significantly (p=0.023) for Hyp (~74% to ~64%). $SA_{TL}$ as a percent of $SA_{TR}$ again did not change for Norm (~23%), but increased significantly (p=0.019) for Hyp (~25% to ~34%). However, $SA_{Lb}$ as a percent of $SA_{TR}$ increased significantly for both Norm (p=0.001; ~0.5% to ~1.6%) and Hyp (p<0.001; ~0.6% to ~2.0%) over the developmental period observed. Furthermore, the slope of $SA_{TL}$ as a percent of $SA_{TR}$ was significantly (p<0.05) higher for Hyp ($b=3.7\times10^{-4}$) than for Norm ($b=0.9\times10^{-4}$), but no difference in the slopes of $SA_{C}$ or $SA_{Lb}$ as percents of $SA_{TR}$ was found between rearing groups.

4.4. Discussion

4.4.1. Whole Body Size and Shape

A number of teleost fishes respond to chronic hypoxia with significant declines in growth rates, but this appears to be dependent upon a number of factors, including the level and duration of hypoxia experienced. Larval char (*Salvelinus alpines*), for example, exposed to chronic hypoxia (~5% $O_2$) for 47 d post-hatch weighed 20% less than their normoxic cohorts (McDonald and McMahon, 1977). The growth rate of Atlantic cod (*Gadus morhua*) also declined when larvae were exposed to chronic hypoxia (~10% $O_2$), likely due to decreased food ingestion rates (Chabot and Dutil, 1999). Juvenile Atlantic menhaden (*Brevoortia tyrannus*) and spot (*Leiostomus xanthurus*) show similar reduced growth rates in extreme chronic hypoxia (4-5% $O_2$), but growth was generally unaffected at higher levels of $PO_2$ (McNatt and Rice, 2004). Thus, the lack of significant difference in body length and larger body mass between Norm and Hyp in developing blue gourami (Figures 4.2, 4.3) is likely due to the higher $O_2$ level (13%) chosen for this study. It would seem reasonable to predict that more
extreme levels of hypoxia (i.e. lower PO$_2$) would result in significant declines in growth rate for this species over the extended period of development observed in this study. Additionally, the lack of significance in SA$_C$ between Norm and Hyp (Figure 4.4) confirms that this level of hypoxia was not adequate to induce long-term gross morphological changes in whole body size or shape. However, as observations of SA$_C$ were only for juvenile gouramis (>30 dpf), it may be that differences do exist in earlier stages, when cutaneous respiration plays a more important role in oxygen uptake. The reduced slope of body length for Hyp at mass$\leq$12mg (Figure 4.3) indicates that Hyp is gaining more mass than length early in development, and this may be indicative of an early shift from cutaneous to branchial respiration.

4.4.2. Gill Development

The decreased elevation of gill arch length at GA-III and lower slope at GA-IV for Hyp (Figure 4.6-A) was surprising, as it was hypothesized that Hyp would show an increased growth rate at the first two gill arches, since these are largely responsible for aquatic oxygen uptake. The decreased elevation of gill arch length at GA-V for Hyp was expected, but the significantly higher slope at GA-VI for Hyp was not. It was alternatively hypothesized that these gill arches would have reduced growth rates under chronic hypoxia, as in Anabantid fishes these arches serve as blood shunts to prevent branchial oxygen loss to hypoxic waters. Thus it may be that the more important role of the gill arches, per se, is one other than respiration (e.g. ionoregulation, osmoregulation), and that the changes observed in Hyp were concomitant with an altered need for such function.
Filament length at GA-III was unaffected by chronic hypoxia, but that at GA-IV of Hyp had an increased elevation (Figure 4.6-B), which was expected due to the role of this arch in gas exchange. While no difference was observed for filament length at GA-V, the slopes of filament length and number of filaments at GA-VI were significantly greater than for Norm. This was unexpected, as the role of GA-VI as a blood shunt would presumably preclude the expansion of branchial structures that could lose oxygen to the hypoxic environment. Similar to the gill arches, this may be indicative an alternate role of the filaments, beyond that of respiration. The lack of significant differences in total number of filaments per animal (Figure 4.7-B), filament density (Figure 4.8), total number of lamellae per animal (Figure 4.9-B), and lamellar density (Figure 4.10) between groups suggests that for blue gouramis in these studies the basic components of branchial structures are rather fixed in development. Thus the actual number or density of filaments or lamellae does not appear to be responsible for environmentally-induced changes in branchial structures of blue gouramis developing under such conditions, but that significant structural modifications owe to alterations in shape of these structures, including length (Figure 4.6), surface area (Figures 4.11, 4.12, 4.13), and possibly thickness of the blood-water diffusion distance.

The slope of SA_{SL} of Hyp was significantly increased at GA-III (Figure 4.11-A), which was in agreement with the hypothesis that increased growth of branchial structures directly responsible for gas exchange (i.e. secondary lamellae of the first two gill arches) would occur in chronic hypoxia. However, the increased SA_{SL} at GA-V was less well understood. Perhaps this arch is more responsible for gas exchange, and plays less of a role as a blood shut, than was previously thought. The significantly lower
slope of $SA_{SL}$ at GA-VI for Hyp was consistent with the hypothesis that the functional respiratory surface area of GA-VI would be reduced in hypoxia, so as to prevent loss of oxygen to the hypoxic water. Although there was a significantly increased slope for Hyp of $SA_L$ only at GA-III, the trend of increased $SA_L$ at all gill arches resulted in a significant increase in $SA_{TL}$ of Hyp (Figure 4.11-B).

Increased surface area of the gills appears to be a common response of fishes to hypoxia. For example, $SA_{SL}$ of larval char exposed to chronic hypoxia for 47 d post-hatch was 38% greater than the normoxic group (McDonald and McMahon, 1977). The total gill surface area of the African cichlid *Pseudocrenilabrus multicolor victoriae* reared for ~5 months in hypoxia (~3% $O_2$) was ~18% greater than that of fish reared in normoxia (Chapman et al., 2000), which is similar to the 14% increase observed in mass-specific $SA_{TL}$ of Hyp in the present study. There was also an inverse relationship between gill surface area of sea bass (*Dicentrarchus labrax*) and the partial pressure in which fish were reared for 3 months (Saroglia et al., 2002). For blue gourami reared in chronic hypoxia, increased $SA_{TL}$ indicates not only that the gills are plastic in their development, but suggests that this species continues to invest energy in methods that maximize oxygen uptake via branchial respiration, even after air breathing has commenced.

4.4.3. Labyrinth Organ Development

Another notable finding in this study is the increased slope of $SA_{LB}$ (Figure 4.13) and 36% increase in mass-specific $SA_{LB}$ of Hyp over that of Norm. The increased growth rate of the labyrinth organ suggests its importance for gouramis developing in
chronic hypoxia. This makes sense from an evolutionary standpoint, as it would be advantageous for larvae exposed to hypoxia to develop their air breathing organ as quickly as possible in order to escape the stress that aquatic hypoxia presents. It is also possible that the labyrinth, in association with the highly vascularized surrounding suprabranchial chamber, plays an earlier role in aquatic respiration prior to the complete transition to air breathing. The structure and composition of the labyrinth organ would not seem to be conducive to reversible remodeling, as has been observed in the gills of some fishes (Sollid and Nilsson, 2007).

4.4.4. Total Respiratory Surface Area

Despite the significant increases in $SA_{TL}$ and $SA_{Lb}$ of Hyp, there was no difference in $SA_{TR}$ between rearing groups (Figure 4.14-A). This can be explained by taking into consideration the large contribution of $SA_C$ to $SA_{TR}$, and the smaller contributions of $SA_{TL}$ and $SA_{Lb}$ (Figure 4.14-B): the slight, albeit insignificant, reduction of $SA_C$ in Hyp likely countered the significant increases in $SA_{TL}$ and $SA_{Lb}$. Additionally, the volume of the suprabranchial chamber was not taken into consideration in this study, and would have possibly altered $SA_{TR}$ between groups.
4.5. Summary

The blue gourami is a freshwater fish native to Southeast Asia, where it generally occupies shallow pools that experience routine and severe hypoxia. Despite the significance placed on aquatic hypoxia as a driving force in the evolution of air breathing in vertebrates, this is the first known study to investigate the morphological effects of rearing an air breathing fish in chronic hypoxia. As selection pressures exist for not only adults, but for every life history stage, it is important to understand the extent to which developmental plasticity can be induced by environmental factors.

While overall growth (total body length, wet mass, cutaneous surface area) of blue gouramis did not differ between rearing groups, a number of morphological modifications of the respiratory system were observed for gouramis developing in chronic hypoxia. Most notably, the hypoxic group had significantly greater growth rates for individual lamellar surface area, total body lamellar surface area, and labyrinth surface area than did the normoxic group.

While the immediate ramifications of such respiratory plasticity can be discerned for individuals, the evolutionary implications of these findings are more elusive. Although this species evolved in response to aquatic hypoxia, the fish used in this study are a domesticated variety, and likely have not been exposed to any marked degree of hypoxia in an unknown number of generations. Thus the “normal” morphological response of gouramis to hypoxia, as would be found in offspring of wild-caught fish, may be radically different than the developmental plasticity observed here. It would be interesting to determine the extent to which epigenetic effects influence respiratory development in this species, especially with reference to hypoxic exposure.
Table 4.1. Summary statistics for whole body measurements of blue gouramis reared in normoxia through 90 dpf. See Table 2.1 for list of abbreviations. NS: not significant.

<table>
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<th>Rearing Group</th>
<th>( L_{TB} )</th>
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<th>( S_{AC} )</th>
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<td><strong>Normoxia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Av. body mass: 94±14.6 mg</td>
<td>1-90 dpf: ( Y=1.74+0.20 ) dpf ( r^2=0.784; n=333 ) ( p&lt;0.001 )</td>
<td>1-90 dpf: ( Y=3.27-0.72 ) dpf+0.03 dpf ( r^2=0.466; n=215 ) ( p&lt;0.001 )</td>
<td>1-90 dpf: ( Y=107.9±13.72 ) mm(^2) ( n=14 )</td>
</tr>
<tr>
<td></td>
<td>( M_{TB}\leq 12 ) mg: ( Y=4.15+0.707 M_{TB} ) ( r^2=0.761; n=142 ) ( p&lt;0.001 )</td>
<td></td>
<td>( M_{TB}: Y=35.84+0.810 M_{TB} ) ( r^2=0.955; n=14 ) ( p&lt;0.001 )</td>
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<tr>
<td></td>
<td>( M_{TB}&gt;12 ) mg: ( Y=10.68+0.066 M_{TB} ) ( r^2=0.859; n=74 ) ( p&lt;0.001 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Av. body mass: 89±24.0 mg</td>
<td>1-90 dpf: ( Y=1.44+0.216 ) dpf ( r^2=0.778; n=90 ) ( p&lt;0.001 )</td>
<td>1-90 dpf: ( Y=8.40-1.04 ) dpf+0.03 dpf ( r^2=0.567; n=80 ) ( p&lt;0.001 )</td>
<td>1-90 dpf: ( Y=-93.1+3.36 ) dpf ( r^2=0.539; n=10 ) ( p=0.016 )</td>
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<td>( M_{TB}\leq 12 ) mg: ( Y=4.14+0.545 M_{TB} ) ( r^2=0.919; n=51 ) ( p&lt;0.001 )</td>
<td></td>
<td>( M_{TB}: Y=35.55+0.761 M_{TB} ) ( r^2=0.964; n=10 ) ( p&lt;0.001 )</td>
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<td>( M_{TB}&gt;12 ) mg: ( Y=11.36+0.064 M_{TB} ) ( r^2=0.849; n=37 ) ( p&lt;0.001 )</td>
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<tr>
<td><strong>Normoxia vs. Hypoxia</strong></td>
<td>1-90 dpf: NS</td>
<td>1-90 dpf: NS</td>
<td>1-90 dpf: NS</td>
</tr>
<tr>
<td></td>
<td>( M_{TB}\leq 12 ) mg: p&lt;0.005</td>
<td>( M_{TB}: NS )</td>
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</tr>
<tr>
<td></td>
<td>( M_{TB}&gt;12 ) mg: NS</td>
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Table 4.2. Summary statistics for gill measurements of gouramis reared in normoxia through 90 dpf. See Table 2.1 for list of abbreviations. III-VI indicates gill arch position. NS: not significant.

<table>
<thead>
<tr>
<th>Rearing Group</th>
<th>$L_{GA}$</th>
<th>$L_F$</th>
<th>$C_F$</th>
<th>$D_F$</th>
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<tbody>
<tr>
<td><strong>Normoxia</strong></td>
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<tr>
<td>Av. body mass:</td>
<td>94±14.6 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III: $Y=1.93+0.009 M_{TB}$</td>
<td>$r^2=0.914$; $n=17$</td>
<td>$r^2=0.827$; $n=17$</td>
<td>$r^2=0.872$; $n=17$</td>
<td>$r^2=0.371$; $n=17$</td>
</tr>
<tr>
<td>IV: $Y=1.52+0.008 M_{TB}$</td>
<td>$r^2=0.915$; $n=17$</td>
<td>$r^2=0.840$; $n=17$</td>
<td>$r^2=0.756$; $n=17$</td>
<td>$r^2=0.001$; $n=17$</td>
</tr>
<tr>
<td>V: $Y=1.06+0.011 M_{TB}$</td>
<td>$r^2=0.858$; $n=17$</td>
<td>$r^2=0.685$; $n=17$</td>
<td>$r^2=0.858$; $n=17$</td>
<td>$r^2=0.687$; $n=17$</td>
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<tr>
<td>VI: $Y=1.09+0.003 M_{TB}$</td>
<td>$r^2=0.687$; $n=12$</td>
<td>$r^2=0.11+0.028$ mm</td>
<td>$r^2=0.986$; $n=9$</td>
<td>$r^2=0.371$; $n=9$</td>
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<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
<td></td>
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<tr>
<td>Av. body mass:</td>
<td>89±24.0 mg</td>
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<tr>
<td>III: $Y=1.89+0.001 M_{TB}$</td>
<td>$r^2=0.886$; $n=12$</td>
<td>$r^2=0.890$; $n=12$</td>
<td>$r^2=0.890$; $n=12$</td>
<td>$r^2=0.890$; $n=12$</td>
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<td>IV: $Y=1.54+0.007 M_{TB}$</td>
<td>$r^2=0.913$; $n=12$</td>
<td>$r^2=0.860$; $n=12$</td>
<td>$r^2=0.856$; $n=12$</td>
<td>$r^2=0.856$; $n=12$</td>
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<tr>
<td>V: $Y=1.18+0.005 M_{TB}$</td>
<td>$r^2=0.939$; $n=12$</td>
<td>$r^2=0.870$; $n=12$</td>
<td>$r^2=0.869$; $n=12$</td>
<td>$r^2=0.869$; $n=12$</td>
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<tr>
<td>VI: $Y=0.84+0.004 M_{TB}$</td>
<td>$r^2=0.898$; $n=11$</td>
<td>$r^2=0.761$; $n=7$</td>
<td>$r^2=0.786$; $n=9$</td>
<td>$r^2=0.844$; $n=9$</td>
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C$_{TF}$: $Y=261+0.9 M_{TB}$

C$_{TF}$: $Y=23-0.02 M_{TB}$

C$_{TF}$: $Y=23-0.02 M_{TB}$

C$_{TF}$: $Y=23-0.02 M_{TB}$
Table 4. 2. (Continued)

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<th>$L_F$</th>
<th>$C_F$</th>
<th>$D_F$</th>
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<td><strong>Normoxia vs. Hypoxia</strong></td>
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</tr>
<tr>
<td>III: NS</td>
<td>III: NS</td>
<td>III: NS</td>
<td>III: NS</td>
<td>III: NS</td>
</tr>
<tr>
<td>IV: p&lt;0.05</td>
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<td>IV: NS</td>
<td>IV: NS</td>
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<td>V: NS</td>
<td>V: NS</td>
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<tr>
<td>VI: p&lt;0.025</td>
<td>VI: p&lt;0.025</td>
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<td>VI: p&lt;0.05</td>
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<td>$C_{TF}$: NS</td>
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Table 4.3. Summary statistics for additional gill measurements of gouramis reared in normoxia through 90 dpf. See Table 2.1 for list of abbreviations. III-VI indicates gill arch position. NS: not significant.

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<tr>
<th>Rearing Group</th>
<th>C&lt;sub&gt;L&lt;/sub&gt;</th>
<th>D&lt;sub&gt;L&lt;/sub&gt;</th>
<th>S&lt;sub&gt;ASL&lt;/sub&gt;</th>
<th>S&lt;sub&gt;L&lt;/sub&gt;</th>
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<tbody>
<tr>
<td><strong>Normoxia:</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Av. body mass:</strong></td>
<td>94±14.6 mg</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>III: Y=606+7.4 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.898; n=17 &amp; p&lt;0.001</td>
<td>III: Y=69+2.4 lam &amp; n=17; (NS)</td>
<td>III: Y=0.0036+2.3e-4 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.880; n=11 &amp; p&lt;0.001</td>
<td>III: Y=1.65+0.074 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.834; n=11 &amp; p&lt;0.001</td>
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<tr>
<td>IV: Y=499+4.8 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.859; n=17 &amp; p&lt;0.001</td>
<td>IV: Y=70-0.06 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.832; n=17 &amp; p=0.012</td>
<td>IV: Y=0.0045+2.4e-4 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.836; n=11 &amp; p&lt;0.001</td>
<td>IV: Y=1.48+0.059 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.901; n=11 &amp; p&lt;0.001</td>
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<td>V: Y=276+2.9 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.859; n=17 &amp; p&lt;0.001</td>
<td>V: Y=64±1.6 lam &amp; n=17; (NS)</td>
<td>V: Y=0.0037+1.0e-4 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.748; n=11 &amp; p&lt;0.001</td>
<td>V: Y=0.96+0.020 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.757; n=11 &amp; p&lt;0.001</td>
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<tr>
<td>VI: Y=193+31.9 lam &amp; n=5; (NS)</td>
<td>VI: Y=57±3.9 lam &amp; n=5; (NS)</td>
<td>VI: Y=0.0022+7.6e-5 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.831; n=5 &amp; p=0.031</td>
<td>VI: Y=0.63±0.189 mm&lt;sup&gt;2&lt;/sup&gt; &amp; n=6; (NS) &amp; S&lt;sub&gt;ALT&lt;/sub&gt; L: Y=5.7+0.32 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.951; n=11 &amp; p&lt;0.001</td>
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<td>C&lt;sub&gt;TL&lt;/sub&gt;: Y=2864+30 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.906; n=17 &amp; p&lt;0.001</td>
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<tr>
<td><strong>Hypoxia:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Av. body mass:</strong></td>
<td>89±24.0 mg</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>III: Y=621+6.9 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.884; n=10 &amp; p&lt;0.001</td>
<td>III: Y=68±0.06 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.657; n=10 &amp; p=0.004</td>
<td>III: Y=0.0033+3.86e-5 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.934; n=8 &amp; p&lt;0.001</td>
<td>III: Y=0.64+0.119 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.948; n=8 &amp; p&lt;0.001</td>
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<tr>
<td>IV: Y=475+5.2 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.904; n=10 &amp; p&lt;0.001</td>
<td>IV: Y=59±2.9 lam &amp; n=10; (NS)</td>
<td>IV: Y=0.0058+2.08e-5 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.847; n=8 &amp; p=0.001</td>
<td>IV: Y=1.86+0.068 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.934; n=8 &amp; p&lt;0.001</td>
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<tr>
<td>V: Y=287+2.4 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.878; n=10 &amp; p&lt;0.001</td>
<td>V: Y=65±0.06 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.686; n=10 &amp; p=0.004</td>
<td>V: Y=0.0032+1.90e-5 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.960; n=8 &amp; p&lt;0.001</td>
<td>V: Y=0.61+0.024 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.958; n=8 &amp; p&lt;0.001</td>
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<td>VI: Y=94±2.0 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.876; n=8 &amp; p&lt;0.001</td>
<td>VI: Y=66±1.7 lam &amp; n=8; (NS)</td>
<td>VI: Y=0.003+1.48e-4 mm&lt;sup&gt;2&lt;/sup&gt; &amp; n=6; (NS)</td>
<td>VI: Y=0.30+0.006 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.808; n=6 &amp; p=0.015</td>
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<tr>
<td>C&lt;sub&gt;TL&lt;/sub&gt;: Y=2730+34 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.929; n=8 &amp; p&lt;0.001</td>
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<td></td>
<td>S&lt;sub&gt;ALT&lt;/sub&gt; L: Y=6.36+0.417 M&lt;sub&gt;TB&lt;/sub&gt; &amp; r&lt;sup&gt;2&lt;/sup&gt;=0.981; n=6 &amp; p&lt;0.001</td>
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\[ Y = \alpha + \beta \text{measure} + \epsilon \]
Table 4.3 (Continued)

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<tr>
<th>Rearing Group</th>
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<th>SA&lt;sub&gt;L&lt;/sub&gt;</th>
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<td><strong>Normoxia vs. Hypoxia</strong></td>
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<td>III: NS</td>
<td>III: NS</td>
<td>III: p&lt;0.001</td>
<td>III: p&lt;0.001</td>
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<td>IV: NS</td>
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<td>V: p&lt;0.01</td>
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<td>V&lt;sub&gt;L&lt;/sub&gt;: SA&lt;sub&gt;TL&lt;/sub&gt;: p&lt;0.001</td>
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<td>C&lt;sub&gt;TL&lt;/sub&gt;: NS</td>
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Table 4.4. Summary statistics for labyrinth surface area (SA$_{Lb}$) and total respiratory surface area (SA$_{TR}$) of blue gouramis reared in normoxia through 90 dpf. NS: not significant.

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<th>Rearing Group</th>
<th>SA$_{Lb}$</th>
<th>SA$_{TR}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>$Y = -0.481 + 0.0219 \ M_{TB}$</td>
<td>$Y = 40.5 + 1.19 \ M_{TB}$</td>
</tr>
<tr>
<td>Av. body mass:</td>
<td>$r^2 = 0.950; n = 18$</td>
<td>$r^2 = 0.986; n = 8$</td>
</tr>
<tr>
<td>94±14.6 mg</td>
<td>$p &lt; 0.001$</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>$Y = -0.347 + 0.0262 \ M_{TB}$</td>
<td>$Y = 39.3 + 1.21 \ M_{TB}$</td>
</tr>
<tr>
<td>Av. body mass:</td>
<td>$r^2 = 0.984; n = 11$</td>
<td>$r^2 = 0.982; n = 7$</td>
</tr>
<tr>
<td>89±24.0 mg</td>
<td>$p &lt; 0.001$</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>Normoxia vs. Hypoxia</td>
<td>$p &lt; 0.025$</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 4.1. Morphological development of gourami larvae reared from <24 h post-fertilization (hpf) through 132 hpf in normoxia (21% O$_2$) and two levels of hypoxia (13% O$_2$ and 10% O$_2$). Note that development of the 13% O$_2$ larvae appears similar to that of the normoxic larvae, whereas gross morphological deformities (indicated with arrows) exist for 10% O$_2$ larvae. No larvae in the 10% O$_2$ group survived past 8 dpf.
Figure 4.2. (A) Total body length and (B) Total body wet mass as function of development of blue gouramis reared in hypoxia through 90 dpf. Solid lines are regressions for Norm data (from Chapter 3). Statistics given in Table 4.1.
Figure 4.3. Total body length as function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regressions with maximized $r^2$ values are shown. Solid lines represent regressions for Norm (Chapter 3). Statistics given in Table 4.1.
Figure 4.4. Cutaneous surface area as function of (A) Development and (B) Total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression lines shown; solid lines indicate mean values (A) or regression (B) for Norm (from Chapter 3). Statistics given in Table 4.1.
Figure 4.5. (A) Gill arch length and (B) Filament length as function of total body wet mass of gouramis reared in hypoxia through 90 dpf. Regression lines shown; solid lines indicate regressions for Norm (from Chapter 3). Statistics given in Table 4.2.
Figure 4.6. (A) Number of filaments by gill arch (filament count) and (B) Total filament count as function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression lines shown; solid lines are Norm regressions (from Chapter 3). Statistics given in Table 4.2.
Figure 4.7. Filament density by gill arch as function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression lines shown where significance occurred; otherwise lines are mean values. Solid lines indicate Norm regressions or mean values (from Chapter 3). Statistics given in Table 4.2.
Figure 4.8. (A) Number of secondary lamellae by gill arch (lamellar count) and (B) Total lamellar count as function of total body wet mass for blue gouramis reared in hypoxia through 90 dpf. Regression lines shown; solid lines are Norm regressions (from Chapter 3). Statistics given in Table 4.2.
Figure 4.9. Lamellar density by gill arch as function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression lines shown where significance occurred; otherwise lines are mean values. Solid lines indicate Norm regressions or mean values (from Chapter 3). Statistics given in Table 4.2.
Figure 4.10. (A) Single lamellar surface area and (B) Total lamellar surface area by gill arch as function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression lines shown where significance determined, otherwise lines are mean values. Solid lines indicate regressions or means for Norm (from Chapter 3). Statistics given in Table 4.2.
Figure 4.11. Total body lamellar surface area as function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression line shown; solid line indicates regression for Norm (from Chapter 3). Statistics given in Table 4.2.
Figure 4.12. Total labyrinth surface area as a function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression line shown; solid line indicates regression of Norm (from Chapter 3). Statistics given in Table 4.3.
Figure 4.13. (A) Total respiratory surface area (SA_{TR}) as function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Regression line shown; solid line indicates regression for Norm (from Chapter 3). Statistics given in Table 4.3. (B) Percent contribution to SA_{TR} by each respiratory structure for Norm and Hyp.
Figure 4.14. (A) Mass-specific surface area of respiratory structures and (B) Total respiratory surface area as a function of total body wet mass of blue gouramis reared in hypoxia through 90 dpf. Solid line in (B) is regression for Norm data (from Chapter 3).
5.1. Introduction

Animals may employ any number of methods to survive and thrive in response to environmental perturbations. In the previous chapter (Chapter 4: Hypoxia-Induced Developmental Plasticity of Respiratory Structures of the Blue Gourami) I showed that blue gouramis developing in hypoxia alter their respiratory morphology, including increases in the surface areas of secondary lamellae and labyrinth organs. I now move on to explore the physiological responses of gouramis reared in hypoxia.

5.1.1. Physiological Responses of Strictly Aquatic Fishes to Hypoxia

Hypoxic exposure of Teleost fishes often results in numerous cardio-respiratory responses to maintain respiratory homeostasis. Strictly aquatic fishes often have increased blood pressure and cardiac stroke volume, which are largely offset by decreased heart rate (bradycardia) (see Farrell, 2007). However, these responses may be dependent upon developmental stage. For example, Jacob et al. (2002) found that zebrafish larvae respond to chronic hypoxia at about the time of hatching by increasing heart rate (tachycardia) as well as cardiac output. By 30 d post-fertilization, however, hypoxic bradycardia is observed (Barrionuevo and Burggren, 1999). There is also a general tendency for increased gill ventilation rate in response to hypoxic exposure, even among developing fishes (Hughes, 1973; Holeton, 1980; Randall, 1982; Vulesevic
et al., 2006). For example, the gill ventilation rate of zebrafish larvae exposed to acute hypoxia (3-4% O₂) increased ~4-fold compared to larvae exposed to normoxia (Jonz and Nurse, 2005). Hypoxic exposure can also result in the redirection of blood from less to more oxygen-dependent organs, as evidenced by the increased perfusion of secondary lamellae of rainbow trout in hypoxia (Booth, 1979).

Fishes can also reveal respiratory plasticity after chronic exposure to hypoxia. These physiological modifications are generally distinguished by a relatively slow onset and by persistence of these changes after removal of the hypoxic stimulus (Powell et al., 1998; Mitchell and Johnson, 2003; Bavis et al., 2007). Plasticity may involve changes in heart rate (Bagatto, 2005) or gill ventilation rate (Burleson et al., 2002; Vulesevic et al., 2006), increased oxygen carrying capacity of the blood (Wood and Johansen, 1972; Schwerte et al., 2003), increased hemoglobin and/or red blood cell concentrations (Schwerte et al., 2003; Timmerman and Chapman, 2004), and heightened resistance to more extreme hypoxic episodes (Rees et al., 2001).

5.1.2. Physiological Responses of Air Breathing Fishes to Hypoxia

The cardio-respiratory responses to acute hypoxic exposure of air breathing fishes differ somewhat from those of strictly aquatic fishes. Increased frequency of air breathing is a generalized response of air breathing fishes to aquatic hypoxia (Lomholt and Johansen, 1974; Fritsche et al., 1993; Mattias et al., 1998; Oliveira et al., 2004; Randle and Chapman, 2005). Aerial hypoxia provokes a similar response. For example, adult blue gouramis responded to both aquatic and aerial hypoxia with a higher rate of air breathing (Burggren, 1979). Adult snake-head fish (*Channa argus*)
(Glass et al., 1986) and pearl gouramis (Trichogaster leeri) (Alton et al., 2007) similarly increased air breathing frequency when exposed to reduced aerial PO$_2$. However, there is considerable variability among air breathing fishes in hypoxia-induced changes in gill ventilation rate, which appear to vary according to the species’ dependence on air breathing. For example, gill ventilation rates of adult obligatory air breathers are generally unaffected by aquatic hypoxia, while facultative air breathers increase aquatic ventilation rates down to the threshold PO$_2$ at which air breathing is initiated (see Graham, 1997).

Air breathing fishes also alter their respiratory physiology in response to long-term hypoxic exposure, but again, not always in the same manner. For example, the walking catfish Clarias mossambicus increased air breathing frequency after being held for 11-13 d in hypoxia (Johnston et al., 1983), while the loricariid catfishes Ancistrus chagresi and Hypostomus plecostomus exposed to hypoxia (~2-3% O$_2$) for 14-21 d showed a reduced frequency of air breathing compared with normoxic cohorts (Graham and Baird, 1982). Similar to strictly aquatic Teleosts, air breathing fishes may also respond to chronic hypoxia with increased hemoglobin concentration and oxygen affinity (Weber et al., 1979; Kind et al., 2002), and possibly even increased oxygen consumption rates (Johnston et al., 1983).

No studies to date could be found that examined the physiological effects of hypoxic exposure on a developing air breathing fish. The goal of this study was to determine if either chronic or acute hypoxia affected heart rate, gill ventilation rate, air breathing frequency, hypoxia resistance, or oxygen consumption rates of developing blue gouramis.
5.2. Materials and Methods

Hypoxic conditions were established, and experimental animals were maintained as described in Section 4.2.1. (Establishment of Hypoxic Conditions). All other aspects of larval care were similar between groups, as described in Chapter 1 (Section 1.2.2. Care and Maintenance of Larvae).

5.2.1. Measurements of Physiological Development

Physiological measurements including heart rate ($f_h$), gill ventilation rate ($f_{GV}$), air breathing frequency ($f_{AB}$) and oxygen consumption ($\dot{V}O_2$) were made as described in Chapter 3. Food was withheld from experimental larvae for 18 to 24 h prior to making any physiological measurements. All physiological measurements in Norm and Hyp larvae were made during exposure to normoxia (21% $O_2$). Additionally, measurements of $f_h$, $f_{GV}$, and $f_{AB}$ for Norm and Hyp were also made while animals were briefly (5-7 min) exposed to moderate hypoxia (13% $O_2$). For these measurements, water was made hypoxic as described above (Section 4.2.1: Establishment of Hypoxic Conditions). For $f_h$ and $f_{GV}$ readings, moderately hypoxic water was circulated through the observation dish (as described in Chapter 2, Section 3.2.1: Heart Rate) at a rate of ~10 ml min$^{-1}$. Additionally, a lid was placed on the observation dish and sealed with putty, and 13% $O_2$ hypoxic gas from the larval holding tank was circulated in the air-space above the water, in order to prevent a normoxic air-water interface. Observations of the onset of air breathing for Norm and Hyp during exposure to extreme hypoxia used the same protocol described in Chapter 3 (Section 3.2.3: Hypoxia-Stimulated Onset of Air Breathing). Additionally, hypoxia resistance was determined by recording the time (s)
from introduction to extreme hypoxia until loss of equilibrium (LOE). During this time, the number of breaths taken was also recorded, and the air breathing frequency \( (f_{AB}; \text{breaths h}^{-1}) \) was calculated. Measurements of \( \dot{V}O_2 \) were made only in initially normoxic water.

5.2.2. Statistical Analyses

Data were analyzed for significant differences between Norm and Hyp, and throughout development within each group using Two-Way ANOVA or a non-parametric alternative, if required. If a significant interaction between Rearing Group and Development (dpf) was found, a post-hoc test (e.g. Holm-Sidak Multiple Comparisons Procedure) was employed. When data were evaluated as a function of body mass, regression analyses were used, and t-tests were utilized to compare the slopes of regression lines between groups. All statistical tests adopted a significance level of \( p=0.05 \), and were analyzed with SigmaStat (Systat) software. Statistical findings are summarized in Table 5.1.

5.3. Results

5.3.1. Heart Rate

Heart rate \( (f_H) \) of hypoxic-reared (Hyp) gouramis measured in normoxia significantly \( (p<0.001) \) increased over the period observed, from \( \sim 120 \text{ beats} \cdot \text{min}^{-1} \) during the embryonic stage (1 dpf) to \( \sim 250 \text{ beats} \cdot \text{min}^{-1} \) at the early larval stage (3 dpf) (Figure 5.1). Heart rate then declined slightly, but remained relatively stable at \( \sim 220 \)
beats · min\(^{-1}\) through 15 dpf. When \(f_H\) of normoxic-exposed Hyp was compared to that of Norm, the \(f_H\) of Hyp was significantly (\(p<0.001\)) lower than that of Norm.

Heart rates of larvae from both groups exposed to moderate hypoxia (13% \(O_2\)) differed significantly (\(p<0.001\)) over the developmental period observed (Figure 5.2-A). Heart rate of Norm increased from 2 dpf (~195 beats min\(^{-1}\)) to 5 dpf (~245 beats min\(^{-1}\)), and then declined significantly by 15 dpf (~215 beats min\(^{-1}\)). Heart rate of Hyp appeared to follow the same trend as Norm, as \(f_H\) increased between 2 dpf (~215 beats min\(^{-1}\)) and 5 dpf (~235 beats min\(^{-1}\)), and then significantly declined through 15 dpf (~205 beats min\(^{-1}\)). When \(f_H\) of larvae exposed to moderate hypoxia was compared between rearing groups, Hyp was significantly (\(p<0.001\)) lower than that of Norm.

5.3.2. Gill Ventilation Rate

Gill ventilation rate (\(f_{GV}\)) of Hyp exposed to normoxia differed significantly (\(p<0.001\)) over the period observed (Figure 5.3). No opercular movements occurred at 1-2 dpf, but \(f_{GV}\) had begun by 3 dpf (~95 opercular beats · min\(^{-1}\)), and then significantly increased between 5 dpf and 9 dpf (~105 beats · min\(^{-1}\)). Thereafter, \(f_{GV}\) remained relatively stable through 15 dpf (~80 beats · min\(^{-1}\)). When \(f_{GV}\) of larvae exposed to normoxia was compared between rearing groups, \(f_{GV}\) of Hyp was significantly (\(p=0.002\)) lower than that of Norm.

Gill ventilation rates of larvae from both groups exposed to moderate hypoxia also increased significantly (\(p<0.001\)) over the developmental period studied (Figure 5.4-A). No opercular movements occurred at 1-2 dpf for either group. Gill ventilation rate of Norm increased significantly by 5 dpf (~270 beats · min\(^{-1}\)), and then declined
slightly to reach ~225 beats · min⁻¹ by 13-15 dpf. Gill ventilation rate of Hyp in moderate hypoxia also increased significantly by 5 dpf (~235 beats · min⁻¹) before slowing to ~175 beats · min⁻¹ by 11-15 dpf. When $f_{GV}$ of larvae exposed to moderate hypoxia were compared between rearing groups, $f_{GV}$ of Hyp was significantly (p=0.001) than that of Norm.

5.3.3. Acute Exposure to Extreme Hypoxia: Onset of Air Breathing

Air breathing for both rearing groups acutely exposed to extreme hypoxia began at 25 dpf (Figure 5.6). Thus, there was no difference in timing of the onset of air breathing between groups.

5.3.3. Air Breathing Frequency

Air breathing frequency ($f_{AB}$; breaths · h⁻¹) of Hyp exposed to normoxia showed significant (p<0.001) differences over the developmental period observed (Figure 5.5). The first air breaths were noted at 25 dpf (~3 breaths ·h⁻¹), followed by an increase in $f_{AB}$ to ~20 breaths · h⁻¹ at 30 dpf. Air breathing frequency peaked by 40 dpf, and then declined to ~10 breaths · h⁻¹ by 60 dpf. No significant difference in $f_{AB}$ measured in normoxia was found between groups over the period observed.

Air breathing frequency of larvae from both groups exposed to extreme hypoxia (~3-5% O₂; aquatic and aerial) similarly increased significantly over the developmental period observed (p<0.001) (Figure 5.6). Air breathing frequency of Norm increased from 0 breaths · h⁻¹ at 5-20 dpf to ~290 breaths · h⁻¹ by 55-60 dpf. For Hyp, $f_{AB}$ increased from 0 breaths · h⁻¹ at 5-20 dpf to ~320 breaths · h⁻¹ by 55-60 dpf. No
significant difference in $f_{AB}$ in extreme hypoxia was found between groups over the developmental period observed. The rate of air breathing attempts in extreme hypoxia represents an average taken over the time to loss of equilibrium (~100-300 s; see Section 5.3.4.), with $f_{AB}$ increasing throughout this time interval.

5.3.4. Hypoxia Resistance

Time to loss of equilibrium (LOE; s) for each group when acutely exposed to extreme hypoxia was significantly ($p=0.003$) different for Norm throughout the developmental period studied (Figure 5.7). Time to LOE for this group tended to increase, from ~115 s at 5 dpf to a peak of ~210 s by 35 dpf, before declining to ~95 s by 55-60 dpf. Significant ($p<0.001$) differences were also found for Hyp over this time, with a pattern similar to that of Norm. Time to LOE for Hyp tended to increase, from ~125 s at 5 dpf to a peak of ~280 s by 35 dpf, before decreasing to ~125 s by 55-60 dpf. When time to LOE was compared between rearing groups, Hyp was significantly ($p<0.001$) greater than Norm throughout developmental period observed.

5.3.5. Oxygen Consumption Rate

Total oxygen consumption rate ($\dot{V}O_2; \mu\text{mol }O_2 \cdot h^{-1}$) for Hyp, calculated from aerial and aquatic $\dot{V}O_2$, was recorded from 1-60 dpf, with data grouped into “bins” of 5 dpf (Figure 5.8-A). The total oxygen consumption rate significantly ($p<0.001$) increased over the time period observed, from ~0.007 $\mu\text{mol }O_2 \cdot h^{-1}$ (1 dpf) to ~0.52 $\mu\text{mol }O_2 \cdot h^{-1}$ (60 dpf). Although no significant differences were observed between any adjacent
grouped dpf, there were significant increases in $\dot{V}_O_2$ between (1-5) dpf and (20-25) dpf, and between (1-15) dpf and (30-60) dpf.

Aquatic $\dot{V}_O_2$ for Hyp showed a similar significant (p<0.001) increase over the time period studied, from $\sim$0.007 µmol O$_2$ · h$^{-1}$ (1 dpf) to $\sim$0.46 µmol O$_2$ · h$^{-1}$ (60 dpf). Again, significant increases were observed between (1-5) dpf and (20-25) dpf, and between (1-15) dpf and (30-60) dpf. Aerial $\dot{V}_O_2$ of Hyp also revealed a significant (p<0.001) increase over the time period observed, from 0 µmol O$_2$ · h$^{-1}$ (1-20 dpf) to $\sim$0.09 µmol O$_2$ · h$^{-1}$ (40 dpf). Significant increases were observed between (1-20) dpf and (40-50) dpf.

When aquatic and aerial $\dot{V}_O_2$ were compared between rearing groups, Hyp was significantly (p<0.005) lower than that of Norm at 50-60 dpf. After the onset of air breathing at 25 dpf, the relative contribution of aquatic respiration to total $\dot{V}_O_2$ declined in Hyp, from $\sim$95% to $\sim$80% by 40-60 dpf, while that of aerial respiration increased from $\sim$5% to $\sim$20% by 40-60 dpf.

Total oxygen consumption rates as a function of body mass (Figure 5.8-B) showed significant (p<0.001) positive relationships for both Norm and Hyp, however the slope of Hyp ($b=0.725$) was significantly (p<0.001) lower than that of Norm ($b=0.767$) on a double log plot.

Mass-specific total oxygen consumption rate ($M_O_2$; µmol O$_2$ · mg$^{-1}$ · h$^{-1}$) of Hyp also differed significantly (p<0.001) over the time period observed (Figure 5.9-A), initially increasing between the embryonic stage (1 dpf, $\sim$0.06 µmol O$_2$ · mg$^{-1}$ · h$^{-1}$) and the early larval stages (5-10 dpf, $\sim$0.13 µmol O$_2$ · mg$^{-1}$ · h$^{-1}$), before declining sharply by 30 dpf ($\sim$0.04 µmol O$_2$ · mg$^{-1}$·h$^{-1}$). When total $M_O_2$ of Hyp was compared to that of Norm, no significant difference was determined.
Aquatic $MO_2$ of Hyp showed a similar trend as total $MO_2$ over the time period studied, with a slight increase between 1 dpf and (5-10) dpf, followed by a significant decline through 30 dpf ($\sim 0.03 \, \mu\text{mol O}_2 \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$). No further significant changes occurred through 60 dpf. Conversely, aerial $MO_2$ of Hyp significantly increased over the time period observed ($p<0.001$), from 0 $\mu$mol O$_2$ mg$^{-1}$ h$^{-1}$ (1-20 dpf) to $\sim 0.009 \, \mu$mol O$_2$ mg$^{-1}$ h$^{-1}$ (40 dpf). Aerial $MO_2$ slightly declined thereafter, but no further significant changes were observed through 60 dpf ($\sim 0.003 \, \mu$mol O$_2$ mg$^{-1}$ h$^{-1}$). No significant difference in aquatic or aerial $MO_2$ occurred between rearing groups.

There was a significant negative relationship for both rearing groups when total $MO_2$ was analyzed as a function of body mass (Figure 5.9-B), with the negative slope of Hyp ($b= -0.27$) significantly ($p<0.001$) greater than that of Norm ($b= -0.23$) on a double log plot.

5.4. Discussion

5.4.1. Heart Rate

Heart rates of both rearing groups measured in normoxia ($\sim 21\% \, \text{O}_2$) followed similar trends (Figure 5.1), but the significantly reduced $f_H$ (bradycardia) of Hyp through 15 dpf indicates that alterations in cardiac function in response to chronic hypoxic exposure occur rather early in development. Furthermore, these changes are sufficiently large such that differences persist even in the absence of a continued hypoxic stimulus. Several previous studies have observed the effects of hypoxic rearing on $f_H$ in fishes, but the results are inconsistent. For example, zebrafish reared in hypoxia ($\sim 10\% \, \text{O}_2$) through 5 dpf showed a significant increase in $f_H$ (tachycardia) at 4-
and 5 dpf (Jacob et al., 2002), while another study found that zebrafish reared in hypoxia (~3 O₂) through 12 dpf had a decreased f₄ compared to those reared in normoxia (Bagatto, 2005). However, PO₂ of the water in which f₄ was measured was not reported for either of these studies, and thus direct comparisons with the current data are difficult.

The heart rate of both rearing groups was significantly lower when acutely exposed to moderate hypoxia than in normoxia (Figure 5.2-A). Bradycardia appears to be a general response to hypoxia in teleost fishes (see Farrell, 2007), and is triggered by externally located branchial oxygen receptors (Taylor et al., 1985; Burleson et al., 1992; Reid et al., 2006). For example, adult channel catfish (Ictalurus punctatus), including those acclimatized to hypoxia (~10% O₂) for 7 d, show a significant reduction in f₄ in response to acute hypoxia (~7-10% O₂) (Burleson et al., 2002). This response, however, may not occur until later in development. Larval rainbow trout do not show decreased f₄ in response to hypoxic exposure until 8 d post-hatch (Holeton, 1971), and zebrafish do not respond until after 20 dpf (Barrionuevo and Burggren, 1999). The bradycardic reflex response to hypoxia is suggested to be absent in larval Arctic char (Salvelinus alpines) through 47 d post-hatch (McDonald and McMahon, 1977). Rather, larvae reared in hypoxia actually had a significant tachycardia compared to normoxic cohorts, when measured in each group’s respective rearing PO₂ (McDonald and McMahon, 1977). This is a very different response than observed here for gouramis, in which f₄ of Hyp in 13% O₂ water was much reduced when compared to that of Norm in 21% O₂ water.
Blue gourami larvae of both rearing groups appeared to respond to moderate hypoxia with decreased \( f_H \) as early as 2-3 dpf (Figure 5.2-A). Hypoxic bradycardia in these very early stages may be a direct result of reduced cardiomyocyte metabolism (Fritsche and Burggren, 1996; Pelster, 1999), while inhibition via the cardiac branch of the vagus nerve may be responsible later in development (Holeton, 1971; Farrell, 2007). The presumed benefits of hypoxic bradycardia are numerous. Branchial oxygen uptake may increase due to the higher pulse pressure distending the lamellae. This may result in a more even distribution of intralamellar blood flow (Farrell et al., 1980; Taylor and Barrett, 1985), or an increased perfusion of lamellae (Farrell et al., 1979). However, these explanations have been questioned (Farrell, 2007). The fish heart may directly benefit from hypoxic bradycardia. As the fish heart lacks an extensive coronary circulation, a prolonged duration of blood in the lumen of the heart may increase the potential for oxygen diffusion to the cardiac tissue. The reduction in cardiac work, as well as the reduced diastolic blood pressure accompanying hypoxic bradycardia, may also serve to reduce cardiac oxygen demand in hypoxic conditions (Farrell, 2007).

5.4.2. Gill Ventilation Rate

The gill ventilation rates of both rearing groups measured in normoxia followed similar trends over the developmental period observed (Figure 5.3), but that of Hyp was significantly lower than Norm by 7 dpf. This suggests that other methods of oxygen uptake and transport are efficient enough in Hyp by this time, such that when exposed to normoxia, \( f_{GV} \) can decline without significantly affecting metabolic rate, at least prior to 15 dpf (see Section 5.4.7). Possible compensatory changes resulting from chronic
exposure or rearing in hypoxia may include morphological modifications of the respiratory system (e.g. increased lamellar surface area) (McDonald and McMahon, 1977; Chapman et al, 2000; present study, Section 4.3.1.2) as well as numerous physiological responses, such as bradycardia (see Farrell, 2007), increased blood pressure, cardiac output, and/or stroke volume (Jacob et al., 2002; Bagatto, 2005), and increased oxygen carrying capacity, binding affinity, or concentration of hemoglobin (Wood and Johansen, 1972; Powers, 1980; Graham, 1983; Schwerte et al., 2003; Timmerman and Chapman, 2004).

Gill ventilation rates of both rearing groups were significantly higher when measured in moderate hypoxia than in normoxia by 3 dpf (Figure 5.4-B). Although neuroepithelial cells (NECs), responsible for O$_2$-chemoreception in fishes, do not form on the filaments of zebrafish until 5 dpf, NECs are present on gill arches by 3 dpf, concurrent with the onset of the hypoxic (~3% O$_2$) hypervenilatory response (Jonz and Nurse, 2005). While initial ventilation of the gills of gouramis appeared somewhat erratic in normoxia, larvae exposed to hypoxia showed stronger and more coordinated movements. This is consistent with studies of completely aquatic larvae, including rainbow trout (Holeton, 1971), Arctic char (McDonald and McMahon, 1977) and zebrafish (Jonz and Nurse, 2005). However, during exposure to moderate hypoxia, $f_{GV}$ of Hyp was significantly lower than Norm by 7 dpf (Figure 5.4-A). This blunted hyperventilatory response is similar to that reported for adult carp (Cyprinus carpio) exposed to chronic hypoxia (~4% O$_2$) for 4 weeks (Lomholt and Johansen, 1979), and for the hypoxia-acclimated (~4% O$_2$ for 14-21 d) facultative air breathing loricariid catfish Ancistrus chagresi (Graham, 1983). However, hypoxic exposure (~2.5% O$_2$) for 27 d of
the air breathing catfish *Clarias mossambicus* resulted in increased gill ventilation (Johnston et al., 1983).

5.4.4. Onset of Air Breathing

The similarity in the timing of the onset of air breathing between rearing groups (Figures 5.5, 5.6) suggests that the physical interactions between animal and environment may more readily govern the onset of air breathing, rather than the physiological necessity for aerial respiration. As most aquatic bimodally-respiring fishes commence air breathing at ~10 mm total body length (Das, 1927; Mishra and Singh, 1979; Prasad and Singh, 1984), it is likely that this is a critical size for overcoming the water surface tension that would otherwise strand smaller larvae at the surface, and for coping with the added buoyancy resulting from inspired air (Graham, 1997). The onset of air breathing for a number of aquatic air breathing fishes is prior to complete development of the air breathing organ (Bader, 1937; Prasad and Prasad, 1985), and thus the act of aerial respiration *per se* may mediate gene expression regulating further development of this organ (Graham, 1997). As the labyrinth organ not only differentiated, but showed an increased rate of growth in blue gouramis reared in the presence of chronic aerial hypoxia (see Chapter 4), aerial normoxia is obviously not a requirement for development of this air breathing organ.

A caveat to studying the timing of onset of physiological systems is that considerations of developmental versus chronological age must be made to properly assess intraspecific differences. Although whole body measurements (body length, body mass, cutaneous surface area) were similar between groups throughout the
developmental period studied, future research would benefit from a developmental index for the blue gourami. This would allow studies of physiological ontogeny to be related to morphological stages of development, and thus would provide a more detailed framework for investigations of physiological heterokairy (see Spicer and Burggren, 2003; Spicer and Rundle, 2007).

5.4.5. Air Breathing Frequency

The air breathing frequency of Hyp recorded in normoxia followed a similar trend as that of Norm (Figure 5.5), with no significant differences observed between groups. The air breathing frequency of developing gouramis of both groups tended to increase upon exposure to extreme hypoxia (~3-5% O₂), although significant differences did not occur between exposure groups until 50-60 dpf. For the obligatory air breathing catfish _Clarias mossambicus_, f_{AB} in hypoxia (~2-3% O₂) was not affected by short-term (<36 h) exposure, but was significantly reduced following acclimation to hypoxia (~2-3% O₂ for 11-13 d) (Johnston _et al._, 1983). The air breathing frequency doubled for adult gouramis exposed to aquatic hypoxia (~5% O₂), and nearly tripled when exposed to the same level of aerial hypoxia (Burggren, 1979). By 50-60 dpf developing gouramis are likely already obligatory air breathers. The combination of aerial and aquatic hypoxia (~3-5% O₂) in the present study may have imposed a synergistic effect on f_{AB} of 50-60 dpf gouramis, as during this time f_{AB} of both groups in extreme hypoxia increased ~20 fold over measurements in normoxia.

Air breathing frequency was highly variable throughout development and at both observed levels of hypoxia (Figures 5.5, 5.6). This is apparently not simply a limitation
of the present study, as such variation is endemic within the literature. No species-specific rate of air breathing seems to be discernable, with $f_{AB}$ being affected by a number of physical and biological factors (Graham, 1997). Thus the lack of significant differences observed between groups and throughout much of development may be due to this variation, and may not truly reflect real differences as would occur in nature.

5.4.6. Hypoxia Resistance

The time to loss of equilibrium for Norm and Hyp followed a similar trend, but Hyp was consistently elevated above that of Norm (Figure 5.7). This suggests that chronic exposure to moderate hypoxia increases the resistance time in acute severe hypoxia, a phenomenon referred to as preconditioning (see Gamperl and Farrell, 2004; Nilsson and Renshaw, 2004). This phenomenon has been reported for other teleost fishes, including larval Arctic char (McDonald and McMahon, 1977), zebrafish (Rees et al., 2001), and in the rainbow trout heart (Gamperl et al., 2004). For species such as the gourami that can experience frequent and extreme hypoxic conditions throughout their life history, early exposure to mild to moderate hypoxia, as occurs during the monsoon rains of the breeding season, may prepare larvae and juveniles for more extreme levels in the future (Hughes et al., 1973).

That hypoxia resistance tended to increase for both groups through 35 dpf may represent an improved efficiency of aquatic respiration or an elevated capacity for anaerobic metabolism (Burleson et al., 1998; Herbert and Wells, 2001; Wells et al., 2003) as development progressed. The sudden decline in resistance time observed between 35-40 dpf may be indicative of the switch from facultative to obligatory air
breathing. This transition may be associated with a decreased hemoglobin-oxygen affinity (Morris and Bridges, 1994), or with an increased diffusion distance at the secondary lamellae (Mishra and Singh, 1979; Prasad, 1989).

5.4.7. Oxygen Consumption

Total (\(\dot{V}O_2\); Figure 5.8-A) and mass-specific (\(\dot{MO}_2\); Figure 5.9-A) oxygen consumption rates of Hyp followed similar trends as those of Norm. Prior to the onset of air breathing at 25 dpf, all oxygen consumption was via aquatic respiration. After 25 dpf, the relative contribution of aquatic respiration to total \(\dot{V}O_2\) and \(\dot{MO}_2\) declined, while that of aerial respiration increased, similar to the findings for Norm.

The significant differences between groups for \(\dot{V}O_2\) as a function of development (Figure 5.8-A) were likely due to the differences in body mass of animals used for measurements of oxygen consumption, with Norm having a significantly higher median body mass than Hyp. This notion is supported by the lack of significant difference between groups for \(\dot{MO}_2\) when similarly analyzed as a function of development. However, Hyp had a significantly lower slope for \(\dot{V}O_2\) and more negative slope for \(\dot{MO}_2\) as a function of body mass than did Norm. Thus, although \(\dot{V}O_2\) does not differ between groups when considering chronological development, with increased body mass Hyp consumes less oxygen per mg body mass than Norm, suggesting that Hyp animals utilize oxygen more efficiently.

Similarities in \(\dot{MO}_2\), as observed throughout development for blue gouramis, also occur in other teleost species reared in either normoxia or hypoxia. For example, offspring of the African cichlid *Pseudocrenilabrus multicolor victoriae*, collected from
routinely hypoxic waters, were reared in normoxia or chronic hypoxia (~3-5% O₂) for ~5 months, and no difference in routine O₂ was found between groups (Chapman et al., 2000). Similarly, no difference in oxygen consumption was observed for starved juvenile turbot (Scophthalmus maximus) reared for 45 d in either normoxia or two levels of hypoxia (~10% O₂, ~15% O₂) (Pichavant et al., 2000). Chronic hypoxia may increase the efficiency of respiration via morphological modifications of the respiratory system (see Chapter 4), by alterations of cardiac function (heart rate, stroke volume, cardiac output), or aspects of ventilation (aquatic or aerial; frequency, amplitude, tidal volume, duration of breath-hold). However, it appears that in these fishes the costs associated with such increased efficiency are not reduced to the extent that overall metabolism can be significantly suppressed.

5.5. Summary

Aquatic hypoxia is a pervasive factor at all stages of life for tropical freshwater fishes, such as the air breathing blue gourami. This environmental perturbation must be met by an ability to maintain respiratory homeostasis, or death is imminent. The selection pressures of hypoxia are most intense during the completely aquatic phase of the blue gourami’s life history. It is crucial for larvae to possess adaptations that allow them to endure hypoxic conditions, such that they may attain the air breathing habit necessary for survival as an adult. The purpose of this study was to explore the ontogeny of cardio-respiratory responses to acute or chronic hypoxia in developing blue gouramis, from strictly aquatic phases through the transition to bimodal respiration.
Both acute and long-term physiological responses of developing blue gouramis to hypoxia were observed. Acute moderate hypoxic exposure elicited bradycardia in both rearing groups. However, larvae reared in moderate hypoxia (Hyp) also showed a significant bradycardia in normoxia. Although gill ventilation rates were elevated for both groups when measured in acute moderate hypoxia, this hyperventilatory effect was blunted for Hyp. Gill ventilation rates were also reduced for Hyp when measured in normoxia. There was a significant increase in air breathing frequency for both groups when exposed to extreme hypoxia at 50-60 dpf, above that measured in normoxia. Additionally, Hyp had a greater resistance to extreme hypoxia than did Norm. Although $\dot{V}O_2$ was significantly lower for Hyp, there was no difference between rearing groups when $\dot{M}O_2$ was considered as a function of development. However, the slopes of $\dot{V}O_2$ and $\dot{M}O_2$, as functions of total body wet mass, were reduced in Hyp, suggesting that Hyp may utilize energy more efficiently.

The ability of a developing animal to respond to altered environmental factors with modifications of morphology or physiology suggests that genetic mechanisms have evolved to deal with such stress. These mechanisms likely arose early in the evolutionary history of animals, but may not necessarily have played a major role in the survival of certain animals in their natural habitats. As the evolutionary history of the blue gourami includes routine hypoxic exposure, rearing these fish in moderate hypoxia, despite certain limitations inherent in the protocols (see Chapter 6), has both ecological and evolutionary importance. The developmental plasticity observed in the current study confirms that the cardio-respiratory ontogeny of this species is quite malleable in response to chronically altered oxygen levels.
Table 5.1. Summary statistics for physiological ontogeny of blue gouramis reared in normoxia (Norm) or hypoxia (Hyp).

.↑↑ Significant increase; ↓↓ Significant decrease; ↑↓↑↑ Significant increase, followed by significant decrease; NS: No significance.

<table>
<thead>
<tr>
<th>Physiological Parameter</th>
<th>Acute Exposure</th>
<th>Developmental Effect</th>
<th>Rearing Effect</th>
<th>Exposure Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate ($f_H$)</td>
<td>Normoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>Hyp&lt;Norm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate Hypoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>Hyp&lt;Norm</td>
<td>Norm: ↓</td>
</tr>
<tr>
<td>Gill Ventilation Rate ($f_{GV}$)</td>
<td>Normoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>Hyp&lt;Norm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate Hypoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>Hyp&lt;Norm</td>
<td>Norm: ↑</td>
</tr>
<tr>
<td>Air Breathing Frequency ($f_{AB}$)</td>
<td>Normoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Extreme Hypoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>NS</td>
<td>Norm: ↑</td>
</tr>
<tr>
<td>Hypoxia Resistance (Time to LOE)</td>
<td>Extreme Hypoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>Hyp&gt;Norm</td>
<td></td>
</tr>
<tr>
<td>Total $O_2$ Consumption ($\dot{V}O_2$)</td>
<td>Normoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>Hyp&lt;Norm</td>
<td></td>
</tr>
<tr>
<td>Mass-Specific $\dot{V}O_2$ ($M\dot{O}_2$)</td>
<td>Normoxia</td>
<td>Norm: ↑↓↓↓</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1. Heart rate ($f_H$) measured in normoxia as function of development of blue gourami larvae reared in normoxia (Norm) or hypoxia (Hyp) through 15 dpf. Values are mean±1 SE; n values given for Hyp; overall significance for Hyp: $p<0.001$. n and p values for Norm given in Chapter 3. Letters indicate grouped dpf for Hyp that are not significantly different; boxes indicate between-group similarity by dpf.
Figure 5.2. (A) Heart rate \( f_H \) in acute moderate hypoxia of gouramis reared in normoxia (Norm) or hypoxia (Hyp) through 15 dpf. Values are mean±1 SE; n values are same for both groups; overall significance for Norm and Hyp: \( p<0.001 \). Letters (a-c: Norm; α-β: Hyp) indicate within-group dpf not significantly different through development; boxes indicate between-group similarities by dpf. (B) \( f_H \) of Norm and Hyp in normoxia and moderate hypoxia. Boxes indicate similarity between rearing groups by dpf.
Figure 5.3. Gill ventilation rate ($f_{GV}$) measured in normoxia as function of development of blue gouramis reared in hypoxia through 15 dpf. Values are mean±1 SE; n values given for Hyp; Overall development for Hyp: p<0.001; n and p values for Norm given in Chapter 3. Letters indicate within-group dpf that are not significantly different for Hyp through development; boxes indicate similarity between rearing groups by dpf.
Figure 5.4. (A) Gill ventilation rate ($f_{GV}$) measured in acute moderate hypoxia as function of development of gouramis reared in normoxia (Norm) or hypoxia (Hyp) through 15 dpf. Values are mean±1 SE; n values equal for both rearing groups. Letters indicate within-group dpf not significantly different through development (same for Norm and Hyp); boxes indicate between-group similarities for acute exposure by dpf. (B) $f_{GV}$ of Norm and Hyp measured in normoxia and moderate hypoxia.
Figure 5.5. Air breathing frequency ($f_{AB}$) measured in normoxia (aquatic and aerial) as function of development of blue gouramis reared in normoxia (Norm) or hypoxia (Hyp) through 60 dpf. Values are mean±1 SE; n values given for each grouped dpf for Hyp; n values for Norm given in Chapter 3. Overall significance for Hyp: p<0.001; no significant differences between median values within Hyp through development, or between rearing groups at any grouped dpf.
Figure 5.6. (A) Air breathing frequency ($f_{AB}$) in extreme hypoxia (3-5% $O_2$; represents “stressed” condition maintained until loss of equilibrium, ~100-300 s) as function of development of gouramis reared in normoxia (Norm) or hypoxia (Hyp). Values are mean±1 SE; n values given same for both rearing groups; overall significance for Norm and Hyp: p<0.001. No significant difference between rearing groups occurred. Letters indicate within-group similarity by dpf for both rearing groups. (B) $f_{AB}$ in normoxia and extreme hypoxia. Boxes indicate between-group similarity by acute exposure and dpf.
Figure 5.7. Time to loss of equilibrium in extreme hypoxia (3-5% O$_2$; aquatic and aerial) as function of development of blue gouramis reared in normoxia (Norm) or hypoxia (Hyp) through 60 dpf. Values are mean±1 SE; n values given same for each rearing group. Letters indicate within-group dpf that are not significantly different through development (same for Norm and Hyp). No significant difference between rearing groups at any dpf.
Figure 5.8. (A) Total oxygen consumption ($\dot{V}O_2$) as function of development for blue gouramis reared in hypoxia (Hyp). Values are mean±1 SE; n values same for Total, Aquatic, and Aerial data points; overall significance for Hyp: p<0.001. Solid line with error bars represents Norm data, given in Chapter 3. Letters indicate within-group similarity by dpf; boxes indicate no significant difference between rearing groups by dpf. (B) $\dot{V}O_2$ as function of body mass. Regression line and equation given for Hyp. Solid line indicates regression for Norm; Norm data and equation given in Chapter 3.
Figure 5.9. (A) Mass-specific oxygen consumption rate ($\dot{MO}_2$) as function of development for gouramis reared in hypoxia (Hyp). Values are mean±1 SE; n values same for Total, Aquatic and Aerial; throughout development: p<0.001 for all Hyp data sets. Letters indicate within-group similarity by dpf. Solid lines represent data for gouramis reared in normoxia (Norm), given in Chapter 3. Boxes indicate similarity between rearing groups by dpf. (B) $\dot{MO}_2$ as function of body mass for Hyp. Regression line and equation given. Solid line is Norm regression; equation given in Chapter 3.
Air breathing in fishes likely arose as a result of frequent exposure to aquatic hypoxia, as routinely occurs in tropical freshwater habitats (Carter, 1957; Johansen, 1970; Graham, 1997; Graham and Lee, 2004; Flück et al., 2007). Selection pressures are encountered not only by adults, but also for every life history stage, thus it is important to understand how environmental factors shape developing animals. Despite the significance placed on aquatic hypoxia as a driving force in the evolution of air breathing, virtually nothing is known of developmental effects of hypoxia on respiratory ontogeny or the transition to bimodal respiration in any species of air breathing fish.

Among the most notable findings of the present studies are the increased lamellar and labyrinth surface areas of blue gouramis reared in chronic moderate hypoxia (Hyp). While increased gill surface area has been reported for strictly aquatic fishes reared in hypoxia (McDonald and McMahon, 1977; Chapman et al., 2000), this is the first study to show that the air breathing organ of a bimodally respiring fish is also augmented by this developmental perturbation. This would suggest that, beyond its evolutionary influences, environmental hypoxia continues to be an impetus for plasticity of respiratory development in the blue gourami. However, as hypoxia was presented in both the aquatic and aerial phases, it is uncertain which of these was responsible for the increased growth of the labyrinth organ. Additional studies, in which oxygen levels of one or both phases are altered, are necessary to tease apart the intricacies of such morphological responses.
Tradeoffs invariably exist for any alteration of structure or function, and thus the costs of the morphological and physiological changes observed in the present studies should be investigated. For example, while increased lamellar surface area allows for improved gas exchange, a number of problems are associated with larger gills, including increased ion flux that must be balanced by energetically expensive ion pumping, heightened exposure to toxins and pathogens, elevated risk of gill injury and resulting hemorrhage, and reduced capacity for feeding due to less space in the oral cavity (see Nilsson, 2007). Thus, gill surface area should be closely matched to gas exchange requirements of the fish to minimize the cost-to-benefit ratio. Indeed, fish with higher metabolic demands have larger gills than less active species (Gray, 1954; Rombough and Moroz, 1997; Nilsson, 2007).

Increased labyrinth organ and suprabranchial chamber growth may also be associated with costs, including reduced feeding capacity, buoyancy control and maneuverability issues (Peters, 1978; Schuster, 1989), and increased risk of aerial predation owing to greater dependence on air breathing (Kramer and Graham, 1976; Wolf and Kramer, 1987; Herbert and Wells, 2001). Due to the convoluted structure of the labyrinth organ of larger juveniles and adults (Das, 1927; Peters, 1978; Graham, 1997), it is conceivable that expansion of surface area may take place without dramatic increases in the overall size of the organ. Nevertheless, most costs associated with enlargement of the labyrinth organs and suprabranchial chambers would appear to be offset by the benefits to the animal, including increased aerial oxygen uptake and resistance to aquatic hypoxia (see Graham, 1997), increased surface area for carbon dioxide elimination (Burggren and Haswell, 1979), decreased exposure to water-borne
toxins (Kulakkattolickal and Kramer, 1988) and other adverse aquatic conditions, as well as increased communication abilities (Yan, 1998; Schuster, 1989).

Also of interest is whether the morphological alterations observed here can be displayed by adult fish, and if these changes are reversible at any stage of development. Pilot studies indicate that these differences may persist into the adult stage when gouramis are maintained in normoxia or hypoxia throughout development. However, it is unclear if these modifications are possible outside of a developmental context—that is, if adults are capable of reversibly remodeling gill structures in the presence of changing respiratory requirements, as has been observed for other fishes. For example, the crucian carp (*Carassius carassius*) increases gill surface area in elevated temperatures or reduced oxygen levels via apoptosis of an interlamellar cell mass, thus exposing previously embedded lamellae—a process that is completely reversible (Sollid and Nilsson, 2006; Nilsson, 2007). Upon aerial exposure, the air breathing mangrove killifish (*Kryptolebias marmoratus*) reversibly reduces its lamellar surface area by growing the interlamellar cell mass, presumably to stabilize lamellae to prevent them from sticking together (Ong et al., 2007). Future studies should compare adult gouramis reared in normoxia or hypoxia throughout development with those reared in one oxygen level and then chronically exposed to another. This would allow for a distinction between developmental effects and those that are acute and reversible.

The marked bradycardia and increased gill ventilation rates observed when strictly aquatic larvae from either rearing group were briefly exposed to hypoxic water were expected, as these are common responses of Teleost fishes to reduced oxygen levels (Hughes, 1973; Holeton, 1980; Randall, 1982; Vulesevic et al., 2006; Jonz and
Nurse, 2006; Farrell, 2007). However, Hyp larvae also showed reduced heart rate and gill ventilation rate in the absence of a hypoxic stimulus (i.e. in normoxia), which may be indicative of a more comprehensive, long-term respiratory plasticity (Powell et al., 1998; Mitchell and Johnson, 2003; Bavis et al., 2007). Additionally, the similarity of routine oxygen consumption between groups throughout development suggests that metabolic demand does not change for Hyp, but that they are more efficient at oxygen acquisition. Mechanisms may include altered cardiac function or gill ventilation, changes in blood properties, structural modifications of gills and air breathing organs (as observed in the present studies), or functional aspects of the air breathing cycle not considered in the present study (e.g. volume of air breath, duration of breath-hold, vascularization and efficiency of oxygen extraction at labyrinth and suprabranchial chambers). Future studies should investigate heart rate and gill ventilation rate for both rearing groups beyond 15 dpf, identifying changes concomitant with the onset of facultative and obligatory air breathing. Partitioning of oxygen uptake between cutaneous, branchial, and aerial oxygen consumption would also be useful to determine the actual contribution of each respiratory structure. Extended oxygen consumption studies would help to clarify how MO$_2$ changes with PO$_2$, and to determine the critical PO$_2$ of this species throughout development.

As the onset of air breathing was not affected by either rearing or exposure environment, it may be that this behavior is more closely associated with the inability of smaller larvae to successfully overcome water surface tension (Graham, 1997), rather than with the physiological necessity of aerial respiration at this developmental stage. Additionally, for many species of air breathing fish, the switch to bimodal respiration is
prior to complete development of the air breathing organ (Das, 1927; Peters, 1978; Prasad and Prasad, 1985), and it has been suggested that the action of aerial respiration itself may be responsible for the expression of genes that regulate further development of this organ (see Graham, 1997). This is reminiscent of “prosynchronotropy”, in which a system becomes functional prior to being necessary for survival (Burrgren and Territo, 1995; Territo and Burrgren, 1998; Burrgren, 2004). For example, the heart of vertebrate embryos begins beating while the needs of the animal are still capable of being met by diffusion alone—that is, prior to the animal requiring convective transport (Pelster and Burrgren, 1996; Territo and Burrgren, 1998; Territo and Altimiras, 2001). Burrgren (2004) has suggested that the heart beat creates pulsatile pressure that promotes angiogenesis, and thus the beating heart creates a stimulus for development of structures and systems that will be required in the future. To determine if early air breathing is truly representative of prosynchronotropy in developing gouramis, future studies should consider the interplay of the onset and frequency of air breathing, different combinations of altered oxygen levels of the aerial and aquatic phases, extended denial of surface access, and morphological development of the labyrinth organ and suprabranchial chambers.

Hypoxia tolerance has been correlated with lamellar surface area in fishes (Chapman et al., 2000; 2002). Thus, the increased resistance time observed for gouramis reared in moderate hypoxia may at least partially be due to the increased lamellar surface area of this rearing group. However, the phenomenon of preconditioning, in which moderate exposure to an environmental stressor allows better resistance to that stressor when experienced later at more extreme levels (Gamperl and
Farrell, 2004; Nilsson and Renshaw, 2004) also holds true for animals acutely exposed to hypoxia (i.e. outside of a developmental context). Thus physiological modifications also likely play an important role in the increased hypoxia resistance observed for developing gouramis. This preconditioning effect may be a necessary part of the natural life history of this species, as the relatively moderate hypoxia present in the habitat during the breeding season (Hughes et al., 1986) would prepare larvae for more extreme levels of reduced oxygen likely to occur in the future. Additional studies should investigate the difference between “absolute” resistance (aerial and aquatic hypoxia) and “ecological” resistance (aquatic hypoxia with access to normoxic air), and should relate these findings to developmental studies of gas exchange requirements, hemoglobin-oxygen affinity, and the ontogeny of facultative and obligatory air breathing.

The studies presented here represent initial attempts at evaluating the developmental responses of an air breathing fish to hypoxia, and thus are in the vein of basic research. As such, limitations of these protocols should be evaluated for their efficacy in related studies in which ecological or evolutionary significance is the focus. For species such as the gourami that spend the first few days of life under parental care (e.g. in a well-oxygenated bubble nest at the surface of the water), it would be more ecologically realistic to allow embryos and young larvae to remain in this environment throughout early development. After this time, larvae could be exposed to more extreme and fluctuating levels of hypoxia than used in these studies, such as those experienced in their natural habitats. Additionally, these studies exposed larvae and juveniles to both aquatic and aerial hypoxia, despite that aerial hypoxia is an exceedingly rare occurrence in nature for species such as the blue gourami. Here,
however, the relatively fast flow of hypoxic water required to maintain a consistent level of hypoxia throughout the water column and minimize the presence of a normoxic surface layer, would have presented additional experimental factors to be considered (e.g. water flow, swimming speed, etc.), as well as yet another ecologically unrealistic condition, as these fish generally live in stagnant ponds or slow moving streams. Despite these limitations, the present studies form a solid foundation for future investigations into the respiratory ontogeny of an air breathing fish that both has a fascinating life history, and is quite conducive to laboratory research.

The evolutionary history of the blue gourami is one that encourages inquiries of not only the origins of air breathing, but also of the continued implications of aquatic hypoxia on the development and efficacy of bimodality. The interplay between genetics and environment is an important factor to consider for future investigations of morphological or physiological responses to developmental hypoxia. For example, parental exposure to an environmental factor may influence developmental parameters of offspring, including that of respiratory ontogeny. This is evidenced by the increased hypoxia resistance of offspring of zebrafish adults exposed to hypoxia for varying lengths of time prior to breeding (Ho, 2008). Gouramis used in the present studies have been bred and reared in captivity for an unknown number of generations, and as such have likely not been exposed to the routine and intense pressures of hypoxia, as would be experienced in their native habitats. Intraspecific studies of the offspring of gouramis obtained from wild populations, as well as multi-generational exposure studies would be useful for teasing apart the intricacies of genetic influences on the respiratory ontogeny of the blue gourami.
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