A HISTOLOGICAL EVALUATION OF THE DEVELOPMENT OF RESPIRATORY STRUCTURES IN CHANNEL CATFISH (*Ictalurus punctatus*) AND TRA (*Pangasianodon hypophthalmus*)

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ABSTRACT

In the realm of finfish production, Siluriformes is one of the most dominant orders on an international scale. Within the United States the family Ictiluridae is most common, while Pangasiids and Clariids are cultured in Africa, Asia, and parts of Europe. In more tropical climates the capacity for production is exponentially greater than in the U.S. due to the ability for higher stocking densities per unit of area. Representative species from these families have distinct morphologies resulting in the presence or absence of an accessory respiratory organ. Ictalurids such as Channel Catfish (*Ictalurus punctatus*) do not have an accessory respiratory organ however tra (*Pangasianodon hypophthalmus*) possess the advantage to utilize atmospheric oxygen to supplement in periods of low dissolved oxygen. Using histological analysis, we identified critical stages of development in Channel Catfish and Tra at which gill, and accessory respiratory structures develop and become functional. Future investigation of these species utilizing RNA sequencing to determine the levels of expression for genes that are active in gill, swimbladder, and air breathing organs.

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List of Abbreviations

ABO	Air Breathing Organ
CS	Central Septum
DPF	Days Post Fertilization
NC	Notochord
SB	Swimbladder
YS	Yolk Sac

INTRODUCTION

The Order Siluriformes is one of the most taxonomically diverse fish groups with 35 families identified as freshwater inhabitants, and two exclusively marine families (Lévêque et al. 2008; Diogo and Peng 2010; Armbruster 2011). The highest concentration of families occur within the neotropics (Lévêque et al. 2008), and include species from the fossil record. Members of Order Siluriformes have been identified on every continent including Antarctica (Armbruster 2011). Several synapomorphies, both derived and shared, have contributed to the reputation and moniker associated with this clade (Armbruster 2011). Common characteristics include a cylindrical muscular body with a flattened venter, spines on the pectoral and/or dorsal fins, and barbels adjacent to a large mouth (Armbruster 2011). Excluding certain families, (*Amphillidae, Loricariidae,* and *Callichthyidae*), which have a dermis covered in bony plates, the order is largely scaleless (Armbruster 2011). This group is biogeographically diverse and comprises 10.8% of all fish species (Armbruster 2011) with many species having a large role globally as sources of protein and staples in commercial aquaculture (Phan et al. 2009; Phuong and Oanh 2010). The complexity of this group is not limited to biogeography, but morphology and physiology as well, with some species having evolved characteristics that are advantageous for a culture environment.

Respiratory mechanisms of fish are not limited strictly to the use of gills to obtain dissolved oxygen from the water, with some species having developed the capacity for aerial gas exchange (Graham 1997). The evolution of air breathing in fishes arose from the Paleozoic and has evolved independently in both major clades of Osteichthyes (Actinopterygii and Sarcopterygii) numerous times, playing a critical role in the 400-million-year history of this class as well as setting the table for the progression of respiratory structures seen in higher vertebrate

taxa (Graham 1997; Hedrick and Katz 2015). In freshwater habitats, the adaptation to utilize atmospheric oxygen is believed to be a direct reaction to exposure to hypoxic environments in the late Devonian, but the true influence is unknown. (Graham 1997; Lefevre et al. 2014a; Hedrick and Katz 2015). Currently, 49 families containing 450 species have been identified possessing this capability (Graham 1997; Lefevre et al. 2014a). Many representatives belong to families still undergoing classification and identification, therefore the true account may be far greater (Lefevre et al. 2014a).

The Siluriformes air breathing capacity varies from obligate repeated surficial air breathing to facultative, augmentative air breathing (Hedrick and Katz 2015). Obligate air breathers like African lungfish (*Protopterus* spp.) often obtain up to 90% of total oxygen consumption directly from the atmosphere (Hedrick and Katz 2015). Classification of air breathing fish has proven difficult with new discoveries consistently blurring the lines of demarcation (Graham 1997). For example, some of the primitive air breathers like gar (*Lepisosteus* spp.) are described to be facultative but when challenged with high water temperatures and hypoxic conditions they must access the surface repeatedly, effectively transitioning them into obligate air breathers (Hedrick and Katz 2015). An increased rate of aerial ventilation in response to hypoxic conditions may seem straightforward for a fish with the ability to breath air, but there are costs to this mode of respiration (Lefevre et al. 2014c). Accessing the surface for oxygen poses complications that can be detrimental to the survival of an individual in any environment. Increased trips to the surface augments exposure to terrestrial and aerial threats and predators while being energetically costly (Lefevre et al. 2014c). Some species do not increase gill ventilation activity as part of the conversion to air breathing, but instead transition directly to aerial respiration (Coolidge et al. 2007; Hedrick and Katz 2015). It

has also been shown that some species will abandon aerial respiration when disturbed or exposed to threats of predation (Lefevre et al. 2014c).

The gills of some air breathing species that are obligatory air-breathers (*Lepisosteus* spp. and arapima; *Arampima gigas*) are often undersized (Coolidge et al. 2007; Lefevre et al. 2014c; Hedrick and Katz 2015), but in some species like tra (also known as striped catfish; *Pangasianodon hypopthalmus*) that are facultative air breathers under normoxic conditions, possess well-developed gill structures (Browman and Kramer 1985; Liu 1993). Gills play a critical role in gas and ion exchange predominantly as a medium to expel carbon dioxide and ammonia through countercurrent exchange (Lefevre et al. 2011). The extent to which an air-breather uses its gill structures fluctuates by species, but presence of the structure is ubiquitous (Lefevre et al. 2011; Lefevre et al. 2014a). The retention of gill structure and functionality gives way to the conclusion that all air breathing fishes are bimodal in their respiration strategy to some extent, meaning they use both gills and air breathing organs (ABO) (Lefevre et al. 2011; Lefevre et al. 2014a; Lefevre et al. 2014c).

Water chemistry, both in the wild and in a culture setting, can shift rapidly in response to a variety of factors such as harmful algal blooms and phytoplankton die offs (Little and Muir 1987). Air breathers or non-air breathers regardless of respiration strategy must be able to sense the environment to regulate the rate of respiration. Central nervous system receptors, sensitive to fluctuations in pH, carbon dioxide partial pressure (PCO_2), and oxygen partial pressure (PO_2), work independently with peripheral chemoreceptors positioned on gill arches that detect PCO_2 levels to control ventilation of gills and air breathing behavior (Hedrick and Katz 2015). Neural respiratory circuits of the lower brainstem interact with motoneurons which activate musculature of the opercular and branchial cavities, effectively conducting respiratory rhythm within the gills

(Coolidge et al. 2007; Hedrick and Katz 2015). Pathways that induce air breathing activities are well less understood, but still rely on the sensing of partial pressures in the external environment as well as internally (Hedrick and Katz 2015).

A morphological development that links all air breathing species is the presence of an ABO that works concurrently with the cardiovascular and circulatory system to retain homeostasis of oxygen, carbon dioxide, and pH in blood and tissue (Lefevre et al. 2014a). An efficient way to do this is to ingest and store atmospheric air internally in highly vascularized structures (Lefevre et al. 2014a). The most rudimentary form of ABO uses cutaneous respiration through the skin and mucosae in which the animal must access the surface (Lefevre et al. 2014c). Other forms of ABO include a respiratory stomach (*Loricariidae*), a labyrinth organ (*Anabantidae, Helostomatidae, Osphronemidae*), modified suprabranchial chamber (*Osteoglossidae, Channidae, Clariidae*), and a modified swimbladder (SB) (*Pangasiidae, Gymnarchidae, Notopteridae*) among others (Lefevre et al. 2014c).

Regardless of the structure of the ABO, fish must increase their rate of aerial respiration to combat potential tissue damage that could arise in a hypoxic environment. This notion is counterintuitive because the gulping of air and storing it within the body represents a functional conflict (Hedrick and Katz 2015). Any cavity within the body occupied by gases changes the buoyancy of a fish. In most teleosts the regulation of buoyancy is mediated by the swimbladder (Hedrick and Katz 2015; Yoshida et al. 2017) Despite being highly vascularized the internal walls of the swimbladder in non-air breathing species are encrusted with guanine crystals which decrease the permeability of the structure reducing the transport of oxygen to the blood stream (Hedrick and Katz 2015). Two forms of this structure have emerged over millennia of evolution. Physoclistous swimbladders are inflated and deflated by the diffusion of gases through a

structure known as a rete mirabile (Pelster 2004). Physostomous swimbladders inflate and deflate via buccal pumping with passage through a pneumatic duct present in the buccal cavity (Hedrick and Katz 2015) making access to the surface necessary to control net buoyancy (Yoshida et al. 2017).

Air breathing fish such as walking catfish (*Clarias* spp.), snakehead (*Channa* spp.), freshwater swamp eel (*Monopterus* spp.), and striped catfish (*Pangasiid* spp.) are frequently farmed species (Lefevre et al. 2014c). Air breathing species represent a large proportion of the global aquaculture effort; approximately 8% of worldwide production (Lefevre et al. 2014c). This value is greater than the global production of salmonids and consequently represents a large source of protein for human consumption (Lefevre et al. 2014c). The decision to raise species that possess an air breathing organ (ABO) is advantageous for an aquaculturist. A primary benefit of growing a species with the ability to obtain and process atmospheric oxygen is that it diminishes sub-lethal effects, including depressed growth rates, often associated with fishes that are sensitive to low dissolved oxygen (Little and Muir 1987). *If there is a low dissolved oxygen event in a culture setting,* a species with the ability to access atmospheric oxygen allows the culturist more time to respond and deploy countermeasures.

Aquaculture in the United States is dominated by family *Ictaluridae*, represented foremost by channel-blue hybrid catfish (*Ictalurus punctatus* ♀ x *I. furcatus* ♂) and channel catfish (*I. punctatus*) (FAO, 2011). Earthen pond-intensive aquaculture of these species amounts to 68% of the total US domestic finfish production (NOAA, 2015). Catfish production plays significant economic and socioeconomic role in states like Alabama, Arkansas, Mississippi, and Texas with 96% of total sales accounted for by these states (USDA, 2018). As of January 1, 2018, food size catfish were being produced on 19,750 hectares (ha) across the U.S. reaching a

total of over 24,000 ha including fingerling and broodfish production (USDA, 2018). Domestic catfish production reached a peak in 2003 at 300.28 million kilograms processed (FAO 2011; Hanson and Sites 2013; Hanson and Sites 2015) and from that point decreased 55% to 136.077 million kilograms processed in 2014 (Hanson and Sites, 2015). Driving forces for the downsize of domestic production include increased cost of feed, which is the highest variable cost for farmers, as well as competition from imported catfish products from Asia (FAO 2011). In recent years, there has been a rapid shift in the composition of processed frozen catfish fillets sold within the United States, with the change in structure favoring the import market (Hanson and Sites 2013). Domestic processed frozen catfish fillets grew to 124 million pounds by 2005, composing 80% of the market; only 30 million pounds were imported (Hanson and Sites 2013). In contrast, by the year 2012, imported products jumped to 78% of the market share, to a total of 237 million with an equilibrium occurring in 2008 (Hanson and Sites 2013). The tra or striped catfish (*P. hypophthalmus*) has infiltrated the western world as an alternative "white fish" substitute and is growing in popularity amongst the American public (Phan et al. 2009).

The culture of *Pangasiid* spp. in Vietnam began in the early 1960's with basa (*Pangasius bocourti*) and striped catfish (*P. hypophthalmus*) using cage culture techniques or small earthen ponds for grow out (Phuong and Oanh 2010). These species exhibit extensive migration (up to 700 km) during reproductive stages, a unique life history strategy that is reported in other air breathing species such as *Channidae* (Lefevre et al. 2014b). Before the development of modern hatching protocols, seed stock were captured from the Bassac, Mekong, and neighboring Tonle Sap Rivers (Phuong and Oanh 2010; Lefevre et al. 2014c). The Mekong River traverses six countries, and divides into an advanced delta system before it flows into the South China Sea. The Mekong Delta has a great capacity for production of aquatic species principally due to its

vast area (641,350 ha) (Phuong and Oanh 2010). Often referred to as an aquatic breadbasket, this region provided over half of the food volume for the entire country of Vietnam corresponding to 17.5 million metric tons of production (Phan et al. 2009).

The culture of *P. hypopthalmus* became dominant with the development of artificial rearing techniques for the basa (*P. bocourti*) in the late 1990's and then to tra. Within Vietnam's tropical climate, it is common to see water temperatures ranging from 25º C to 30 º C annually, sometimes reaching 34º C in the epilimnion of earthen ponds (Lefevre et al. 2011; Lefevre et al. 2014c). There is an inverse relationship between the temperature of water and the capacity for it to hold oxygen, making hypoxic conditions a common occurrence. The ability to air breath has given striped catfish an advantage in this culture environment and led to a success story in commercial aquaculture.

Although originally classified (under *Pangasius sutchi*) as a continuous obligate air breather (Browman and Kramer 1985), a multivariate respirometry study conducted by Lefevre et al. (2010) concluded that tra are facultative air breathers with a high capacity for aquatic respiration. The swimbladder changes greatly during initial development and over different growth stages (Zheng and Liu 1988). In a morphology and development study Zheng and Liu (1988) concluded that the swimbladder appears at 9-11 days post fertilization (dpf). Later Liu (1993) determined that the SB appears much earlier in development (6 dpf) a small blind tube extending from the dorsal surface of esophagus. At 9-11 dpf the organ enlarges extensively (Liu 1993). The outer walls of this highly elastic structure are comprised of collagen fibrosa (Browman and Kramer 1985; Zheng and Liu 1988; Liu 1993). The inner walls of the developing swimbladder are covered in a layer of cuboidal epithelium which later becomes stratified squamous epithelium and are supported by a dense capillary network (Zheng and Liu 1988; Liu

1993). The At 13-15 days post fertilization the inner walls invaginate and begin to form trabeculated respiratory alveoli (Liu 1993). By 30 DPF the SB has developed into an oval shape with a wide anterior portion tapering slightly to a point at the posterior (Browman and Kramer 1985; Zheng and Liu 1988; Liu 1993). The capillary network has advanced in complexity at this stage along with increased internal surface area. The swimbladder is linked to the Weberian apparatus through the tripus at the posterior end (Zheng and Liu 1988). At 60 DPF the shape of the SB is now lobate at the anterior tapering to a single conical chamber at the posterior (Browman and Kramer 1985; Liu 1993; Zheng and Liu 1988).

The bottom of the anterior lobe is connected to the dorsal surface of the esophagus though a well-developed, broad, short, pneumatic duct (Browman and Kramer 1985; Liu 1993; Zheng and Liu 1988). The pneumatic ducts is formed of mucosa, fibrosa, and muscularis a composition similar to the esophagus with stratified squamous epithelium coating the interior (Zheng and Liu 1988). The swimbladder consists of a mucosa layer containing a surface epithelium and stratum compactum retained within a collagen fiber wall (Zheng and Liu 1988; Liu 1993). A central septum composed of collagen divides the structure along a sagittal plane with the primary artery and vein located within the septum (Zheng and Liu 1988). The septum divides a pair of cylindrical tubes that run the entirety of the swimbladder and open into the anterior lobe forming respiratory alveoli (Browman and Kramer 1985; Zheng and Liu 1988; Liu 1993). At 90 days post fertilization the swimbladder has elongated, and the posterior point enters the ventrolateral musculature. Alveoli have increased within the structure and the interior resembles a sponge like substance (Zheng and Liu 1988; Liu 1993). At 6-12 months post fertilization the respiratory functionality of the organ has greatly improved (Zheng and Liu 1988). The walls of the SB can no longer expand or contract and have thickened significantly

(Zheng and Liu 1988). The SB retains the conical shape tapering to the posterior and extending into the ventrolateral muscle (Browman and Kramer 1985; Zheng and Liu 1988) with an increased number of alveoli maintaining the spongy texture (Zheng and Liu 1988). The respiratory epithelium is 25-30 µm thick and highly vascularized with a rich capillary network (Zheng and Liu 1988).

Artificially reared *P. hypopthalmus* spawn in a tropical climate with optimum water temperatures from 28-31º C when using inducing agents such as carp pituitary extract (CPE), Ovaprim, and Ovatide (Chand et al. 2011). Channel catfish will spawn in water temperatures ranging from 23-30º C with an optimum spawning temperature of 27º C (Wellborn 1988). The differentiating environments of these species plays a direct role in their dissimilar rates of development. Embryonic maturity post fertilization is predominantly a temperature driven variable. At 26º C and above, *Pangasianodon* eggs hatch within 24 hours after fertilization, but with depressed temperatures reaching 20º C hatch can occur up to 12 hours later (Islam 2005).

Unfertilized striped catfish eggs are pliable and round, but upon fertilization they become firm and assume a circular shape with a diameter ranging from 1.2 - 1.8 mm (Islam 2005; Morioka et al. 2009). Upon hatch, the fry range from 2.98-3.2 mm in length with a large yolk sac (Islam 2005; Morioka et al. 2009). By the third day post fertilization the fry have increased in size to 5.45-5.57 mm in length with an advancement in development of the eyes, mouth, and caudal fin (Islam 2005). The yolk sac is completely absorbed by five days post fertilization. At this age the larvae have the capability to swim freely (Islam 2005; Morioka et al. 2009). The gas bladder emerges at this stage between the notochord and peritoneal cavity (Morioka et al. 2009)

Unfertilized channel catfish eggs are large yellow 3.5-4 mm diameter agglutinant spheres (Islam 2005). Temperature dependent hatch ranges from 5-10 DPF (Wellborn 1988). Pre-flexion

larvae have a large yolk sac and survive on endogenous feeding for 2-5 days (Wellborn 1988). The swimbladder of *I. punctatus* is located outside of the peritoneal wall underneath the vertebrae and is situated between both the head and trunk kidneys (Grizzle and Rogers 1976). The physostomous swimbladder of the channel catfish possesses a pneumatic duct located close to the stomach, which connects the ventral portion swimbladder to the esophagus (Grizzle and Rogers 1976). The morphology of a mature swimbladder consists of three chambers, with a transverse septum creating an anterior chamber and a sagittal septum forming two parallel chambers (Al-Rawi 1967; Grizzle and Rogers 1976). The anterior chamber communicates with the posterior lobes through openings in the transverse septum and attaches to the tripus of the Weberian apparatus (Al-Rawi 1967; Grizzle and Rogers 1976). Encasing the swimbladder is a wall composed of fibrous collagenic tissue with the outer wall (tunica externa) containing two layers of connective tissue and the internal layer (tunica interna) composed of squamous epithelium (Grizzle and Rogers 1976).

The ability to air-breathe allows for a great disparity between the capacity for culture per unit area of these species. Phan et al. (2009) found that over 75% of farmers within the Mekong delta reported *P. hypopthalmus* production of 300 metric tons/ha in comparison to channel-blue hybrid catfish that have a ceiling of about 29 metric tons/ha (Bosworth et al. 2015). Channel-blue hybrid catfish and channel catfish, the two primary genetic types used in US catfish farming, employ a physostomous swimbladder which has minimal capacity for gas exchange, but facing a hypoxic event these fish will gulp at the surface attempting to profit from any available oxygen source. This behavior is very similar to the behavior that striped catfish use when accessing atmospheric oxygen. It would be informative to compare these species using histological methods for comparison of critical time points in development.

In this study a protocol was developed to create histological images from catfish larvae using a cryostat microtome. The objective of this analysis was to histologically compare the development of respiratory structures in channel catfish (*I. punctatus*) and tra or striped catfish (*P. hypophthalmus*) at varying stages of development. Although the respiratory swimbladder of *P. hypophthalmus* has been analyzed before (Browman and Kramer 1985; Liu 1993; Zheng and Liu 1988), a fine scale analysis was necessary during the critical transitionary period in which the larvae develop a functioning air breathing organ. Histological data was correlated with oxygen challenge data for the striped catfish generated by collaborators at Can Tho University, Vietnam.

MATERIALS AND METHODS

Experimental channel catfish fish spawning and incubation

Channel catfish embryos and fry were obtained by artificially spawning Kansas random strain of brood stock raised at the Genetics Research Unit, E.W. Shell Research Center, Auburn University. The Kansas strain originated from the Ninnescah River in Pratt, Kansas in 1911 (Smitherman et al. 1983). Channel catfish brood stock were harvested from earthen ponds in early June 2017. Fish were transported to a genetics facility greenhouse in a cylindrical hauler containing water. The salinity of the water was raised to 3 parts per thousand (ppt) by the addition of sodium chloride (NaCl) during transport to reduce the risk of osmoregulatory stress (Francis-Floyd 1993). Dissolved oxygen (DO) was maintained above 5 mg L^{-1}. Labeled mesh laundry bags were used to hold channel catfish females within tanks that maintained dissolved oxygen >5 mg L^{-1} and constant water flow. Artificial spawning was induced in the females by an intraperitoneal injection of liquid luteinizing hormone releasing hormone analog (LHRHa) A priming dose of 20 µg/kg was utilized followed by a resolving dose of 100 µg/kg. Thirty-six hours after the second injection females were examined for ovulations, and if no eggs were present they were continually checked every 4 hours.

At the first sign of ovulation, the females were removed and anesthetized in tricaine methane sulfonate (MS-222) at a concentration of 100 parts per million (ppm) buffered using sodium bicarbonate ($NaC0_3$). The females were then rinsed with water, dried with a towel and manually stripped of eggs into greased spawning pans. Eggs were washed, and blood was removed with and exchange of 0.9% saline solution. The males were euthanized by blunt force trauma to the head executed by an exact strike.

An incision was made on the ventral portion of the body starting at the urogenital pore and the testes were extracted. Any blood or contaminants present were removed manually then the gonads were rinsed using a 0.9% saline solution. The testes were then dried and weighed to the nearest 0.5 g. To make the sperm solution the testes were macerated and diluted with 0.9% saline solution at a rate of 10 ml for each gram of testes extracted from the male to make the sperm solution.

The sperm solution was mixed with the eggs while still in the spawning pan with enough solution to fertilize all the eggs. Water was then applied to activate the gametes and 5-10 minutes were allocated for the eggs to fertilized and congeal. The spawning pan was placed in a hatching trough with a calcium chloride ($CaCl_2$) drip to maintain water hardness above 40 ppm. The egg masses were then gently moved to a basket equipped with a 2 mm square plastic mesh fry catcher. The hatching trough contained a rotating paddle wheel to aerate the egg mass, and a $CaCl_2$ drip. The hatching troughs were checked daily for dissolved oxygen levels so that dissolved oxygen levels of 5 mg L^{-1} were constantly maintained. If fungal infection occurred, a 15-minute static treatment of formalin was administered at a rate of 100 ppm. Treatments were ceased 24 hours prior to expected hatch date. Newly hatched fry were fed a powdered 50% protein starter diet from Purina® AquaMax® starting at 7 days post fertilization. Fish were fed to satiation six times per day.

Sample collection (channel catfish)

One primary egg mass was used for sample collection with a second egg mass that was also Kansas random collected a day later for reserve. The reserve was utilized for a single collection at 1.5 days post fertilization.

Samples were collected every 12 hours for the first five dpf and every 24 hours from 5-30 dpf collected every 24 hours. A total of event 20-50 eggs/embryo/larvae were collected at each sampling. Larvae were euthanized with 200 ppm buffered MS-222. The samples were placed into 15 mL centrifuge tubes containing 10% phosphate buffered formalin and sealed with screw top lids.

Striped catfish samples

Samples for the Tra (*P. hypophthalmus*) were received from collaborators at Can Tho University, Vietnam. The eggs were collected from brood stock and fertilized on November 17, 2016. The samples hatched within 24 hours of fertilization and were sampled every 24 hours for the first 30 days after fertilization.

Challenges to determine the tolerance of *P. hypophthalmus* to low dissolved oxygen were conducted by collaborators at Can Tho University, Vietnam. Larvae were challenged each day from 3-12 days post fertilization. Fifteen-20 larvae were placed in a two-liter container, a separate control group was stocked in a second two-liter container for each experiment. Supplemental oxygen was removed, and the dissolved oxygen level was lowered through chemical manipulation until 0 mg L^{-1} dissolved oxygen was obtained. Dissolved oxygen levels, percent survival and behavior were calculated every 15 minutes.

Cryostat Microtome Sample Preparation

Samples fixed in 10% phosphate buffered formalin were rinsed with reverse osmosis water or ultrapure water with three five-minute exchanges for a total of 15 minutes. The rinsed samples were then transferred to a 15 mL plastic centrifuge tube containing a 30% sucrose solution for 12 – 24 hours, or until the samples sank to the bottom. The tubes were labeled with

the species, age of the sample and date of preparation. The sample was removed from the sucrose solution and the fish were rinsed again for five minutes in reverse osmosis or ultrapure water. At this time, the specimens were trimmed using a curved dissecting forceps and a #22 scalpel. Using the curved forceps, the caudal region of the sample was immobilized with gentle pressure since excessive pressure on the body cavity will damage internal structures. The scalpel was used to create a transverse cut directly posterior to the genital pore. Samples greater than 7 mm required additional trimming, up to 4-5 mm of the posterior, to fit into the cryosectioning mold. The head was never grasped by the forceps to prevent damage to the gills.

Tissue Tek embedment media, (Electron Microscopy Sciences, Washington, PA), was poured into a disposable 10x10x5 mm plastic mold, and the trimmed sample positioned within the media. The mold was placed in a vacuum chamber for 10-15 minutes at 20 (mm HG), to encourage media to infiltrate the branchial cavity and gill structures to improve sectioning. During sectioning, the blade was mounted securely to the blade holder body and locked on to the base of the cryostat chamber. It was critically important for the blade holder body to have the same temperature as that of the sample being cut, to prevent curling of the sections during the cut.

Freezing was completed within the chamber of the microtome (a Reichert-Jüng Frigocut 2800 cryostat, Austria) The cryotome was always run and left to cool for at least two hours to bring it to a stable -22º C, the optimal temperature for freezing and cutting.

A fresh mold was filled with Tissue Tek® and placed on the freezing post within the cryostat for 90 seconds then removed. The sample was gently lifted out of the original mold delicately using forceps and then placed in the chilled mold head down completely covered with the embedment media. The orientation oral-aboral axis was always perpendicular to the base of

the mold so that during sectioning transverse sections were produced. To create different planes of sectioning the sample was placed in a different orientation within the mold. Freezing occurred within 5-8 minutes, but the mold remained within the chamber for at least 10 minutes to ensure the block was frozen solid. At this stage, an ample drop of Tissue Tek® was added to the specimen chuck, which is the piece that fits on the freezing posts within the machine. The frozen block was then popped out of the mold by applying pressure to the back while grasping the sides. Immediately after doing so the frozen block was be placed on the drop of Tissue Tek® on the specimen chuck. The sample was always oriented so that the larvae were sequentially cut from posterior to anterior.

The specimen chuck holding the block was then returned to the freezing chamber of the cryostat for an additional 5-10 minutes. After it was completely frozen, the specimen chuck was removed and trimmed of excess Tissue Tek®. The chuck was then mounted to the carrier. The blade was in a fixed position at this stage and the controls of the cryostat were used to maneuver the carrier close to the blade.

Sectioning the block

The internal temperature of the cryostat chamber was turned to -20 C at this stage for optimal cutting. The blade angle was set to 4º to produce 30 µm sections . Although the cryostat is motorized we found that manual sectioning was necessary to produce reliable results. Once the block was in place, the hand wheel was turned to trim the block until the tissue was exposed enough to start placing sections on the 0.1% gelatin-subbed slides. The handwheel was rotated slowly and did not exceed a rate of 90º every 10 seconds while cutting. While rotating the hand wheel, pressure was also applied to the anti-roll plate so that the frozen sections come out flat. The anti-roll plate was then retracted, and the 0.1% gelatin subbed slides moved into position to

capture the section. Once the slide was aligned with the frozen section, the slide was gently pressed down until the section was attached to the slide and then it was removed with a rolling motion. The Tissue Tek® melted to the slide and the tissue became attached. Four to eighteen sections were placed on the slides depending on the size of the sample. The cutting and adhering of sections to slides was repeated until the whole sample had been cut. Slides were then dried for up to one hour before the staining process.

Staining

After drying the slides containing samples were loaded on an Electron Microscopy Sciences (E.M.S.) Easy Dip staining rack, 10-12 slides per rack. The slide rack was wiped down with a paper towel between each step to remove any transfer to the next solution. Depending on the age (dpf) of the sample, differentiating times were used for immersion in the staining process.

Slides were dipped in 95% ethanol and then transferred to 10% phosphate buffered formalin for fixation. The embedment media is then cleared with 4-5 dips in ultrapure water. The rack is then transferred to the primary stain, filtered Harris hematoxylin, for 1-17 seconds depending on age (Appendix A, Fig 1). In the regression stage, the slides were rinsed in ultrapure water by dipping them 3-5 times. An immersion bath in ultrapure water for 10 seconds followed by a single dip clears the last of the excess stain. The rack was transferred to the final step in the regression, a 10 second bath in 95% ETOH. The slides were moved into a jar of Eosin Y for 10-15 seconds for counterstain (Appendix A, Fig 1). The slide rack was wiped thoroughly of excess stain before moving to the dehydration phase. The slides were moved to a 95% ETOH solution and dipped 3-5 times (Appendix A, Fig 1). Following this bath, the slides were moved to another 95% ETOH solution.

	Step:	1	2	3	4
	Solution:	95% ETOH	10% Phosphate Buffered Formalin	Ultrapure H2O	Filtered Harris Hematoxylin
	Action:	Immerse	Immerse	Dip	Immerse
Age (DPF)					
5 to 7		10 sec	10 sec	4x	1-5 sec
7 to 10		10 sec	10 sec	5x	12 - 15 sec
10 to 25		15 sec	10 sec	5x	15 - 17 sec
Purpose		Fixation		Rinse (clears T.T.)	Primary Stain

	Step:	5	6	7	8
	Solution:	Ultrapure H2O	Ultrapure H2O	95% ETOH	Eosin Y
	Action:	Dip	Immerse	Immerse	Immerse
Age (DPF)					
5 to 7		3x	10 sec	10 sec	10 sec
7 to 10		5x	10 sec	10 sec	15 sec
10 to 25		5x	10 sec	10 sec	15 sec
Purpose		Regression			Counterstain

	Step:	9	10	11	12
	Solution:	95% ETOH	95% ETOH	100% ETOH	100% ETOH
	Action:	Dip	Immerse	Immerse	Immerse
Age (DPF)					
5 to 7		3x	10 sec	5 sec	10 sec
7 to 10		5x	10 sec	5 sec	10 sec
10 to 25		5x	10 sec	5 sec	10 sec
Purpose		Dehydration			

Figure 1: Twelve step regressive Harris hematoxylin and eosin (H.&E.) staining protocol developed for channel catfish (*Ictalurus punctatus*) and tra (*Pangasianodon hypophthalmus*) from 5-25 days post fertilization (dpf) for use with slides made with a cryostat microtome. Younger catfish larvae of both species were more sensitive to the actions of the staining process. Variables that were different between age groups include the initial immersion of 95% ETOH, subsequent dips in ultrapure water, immersion in Harris hematoxylin, eosin y, and the final 95% ETOH dip.

solution and immersed for 10 seconds. The slides were then transferred to a 100% ETOH bath for 5 seconds. The last step in the staining process is an immersion in 100% ETOH for 10 seconds to conclude dehydration

Slide mounting

The slides were dehydrated then mounted using 24 x 60 mm rectangular VWR® Micro Cover Glasses suitable for No. 1 ½ slides. Cover glasses were cleaned using ethanol and a Kimtech® KimWipe™ low lint cloth. The slides were prepped by using a cotton swab to remove any excess stain or Tissue Tek® from around the sections. After allowing the clean slide to dry in a dust free environment, a few drops of Permount mounting medium were added to the surface of the slide. The cover glass was lowered on to the slide by placing one end of the glass on the slide and allowing it to roll on to the slide. This method prevented bubbles from forming in the mounting media.

Imaging

Digital images were created using an Olympus® BHS fluorescence binocular microscope equipped with a 3.4 megapixel color digital camera (Qimaging® model Micropublisher 3.3 RTV). Selected fields were imaged with 10x and high 20x SPlan objectives with a transfer factor of 2.5X. Image Pro Plus 7 (Media Cybernetics, Bethesda, MD) was used to capture the images; ImageJ (v1.51k) was used for annotation. Cell types were identified using a catfish histology manual developed by Grizzle and Rogers (1976).

RESULTS

Ictalurus punctatus

The channel catfish eggs began to hatch at 120 hours post-fertilization. The newly hatched larvae congregated in a mass at the bottom of the fry catcher. Fry were sustained via yolk sac absorption for the first 5 days post hatch then were fed supplemented feed.

At 185.5 degree days a few organs had developed, and the yolk sac encompassed a large portion of the body (Fig 2a,2b). A small cavity formed within the yolk sac that surrounds simple gastrointestinal structures (Fig 2a,2b). Skeletal musculature surrounds the vertebral column with the crest of the dorsal fin present (Fig 2a,2b). The newly emerged swimbladder was located ventral to the notochord and dorsal to the peritoneal cavity (Fig 2a,2b). The structure was laterally compressed at the posterior end with little distinction of the parallel lobes present (Fig 2a) The more anterior section (Fig 2b) revealed the swimbladder was slightly differentiated into two lobes and separated buy a thin central septum (Fig 2b).

At 212 degree days the larvae were developed sufficiently to reach the “swim-up” stage. At this point the larval fish could regulate themselves in the water column and had gained the capability to access the surface for feeding. In 24 hours, the morphology of larvae, revealing moderate increase in the dimensions of the developing internal organs (Fig 3). Much of the body cavity was still comprised of yolk. The pocket surrounding the developing gastrointestinal structures increased in size within the yolk sac

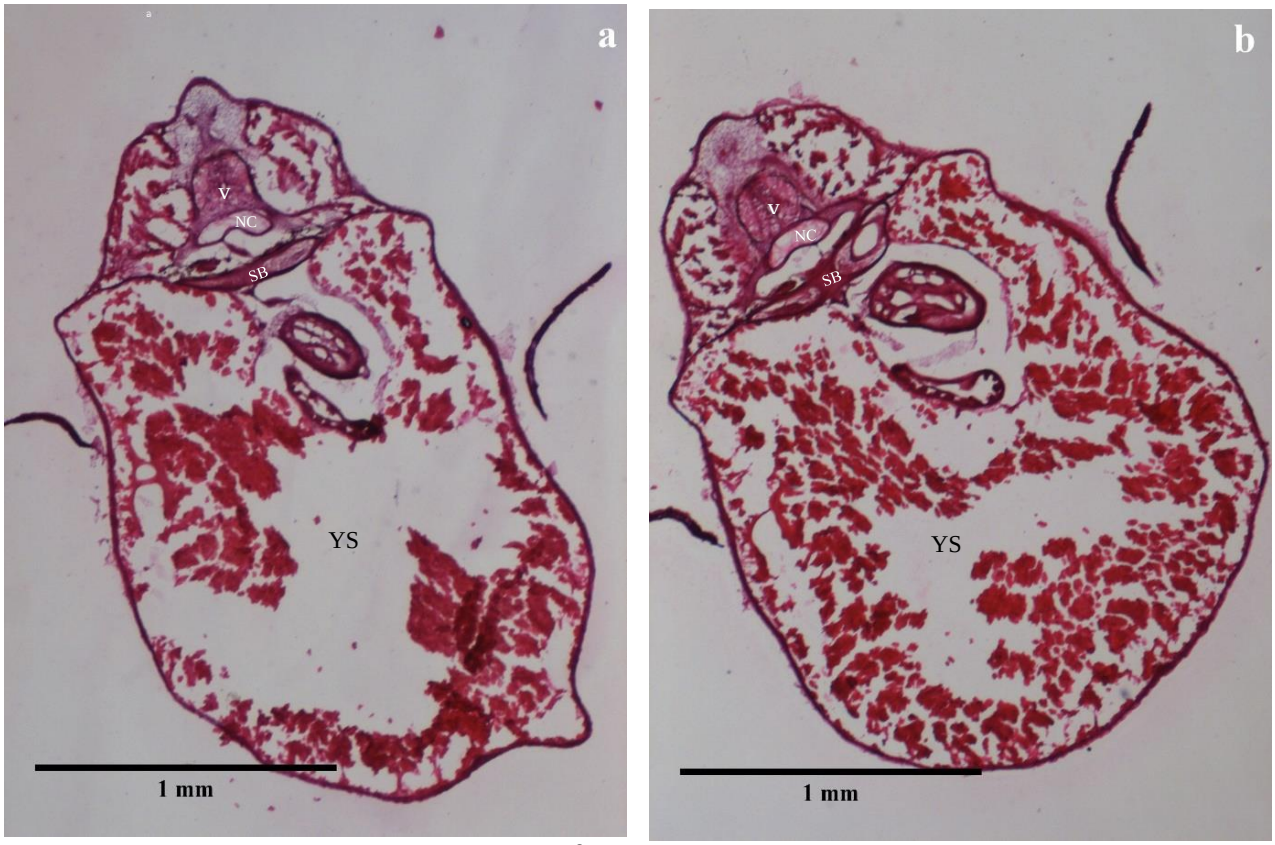

Figure 2: 30µm thick transverse sections of *Ictalurus punctatus* larvae 185.5 degree days showing the internal structure, including the vertebrae (V), notochord (NC), yolk sac (YS) and swimbladder, (SB). Void within the yolk was a result of damage during preparation of slides. (a) Posterior section shows the swimbladder laterally compressed lacking the capacity for gas retention. (b) Anterior lobate portion of the swimbladder.

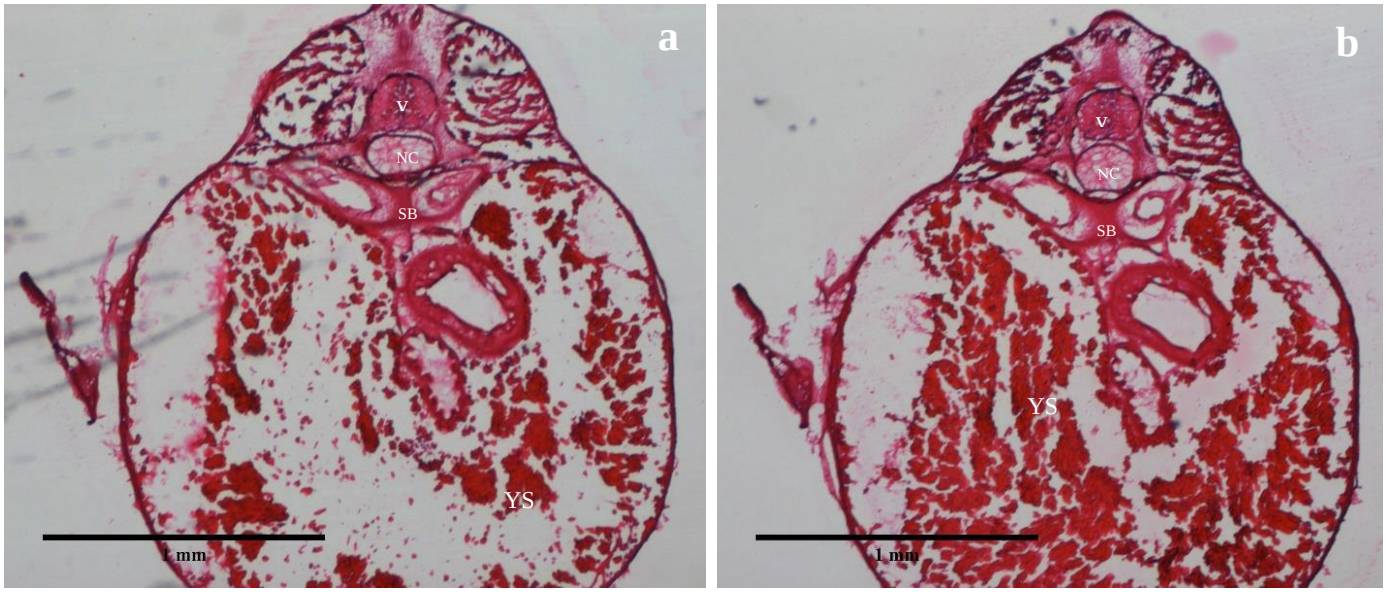

Figure 3: 30µm thick transverse sections of *Ictalurus punctatus* larvae 8 days post-fertilization showing the internal structure including the vertebrae (V), notochord (NC), yolk sac (YS) and swimbladder, (SB). Open area within the yolk was a result of damage during preparation of slides. Section (a) was directly posterior to section (b).

along with the diameter of the tube-like structures. The lobes of the swimbladder had extended toward the dorsal perimeters of the body cavity (Fig 3a,3b) still resting between the muscle layer and the peritoneal cavity. The central septum separating the two posterior lobes was thickened Fig (3a). The internal voids increased in diameter allowing a greater capacity for the retention of gasses. Layers of squamous epithelium had developed into the tunica interna or internal layer of the swimbladder.

The swimbladder and other internal organs developed further at nine days post-fertilization. The diameters of the hollow intestinal structures increased significantly (Fig 4a,4b) when compared to the histological section from the previous day (Fig 3). The yolk decreased in volume and was confined to the outer perimeter of the ventral portion of the body cavity. Counteractively, the organs of the digestive system were increased along with the borders of the cavity containing the organs in comparison to the previous day. The swimbladder increased significantly in size and extended further to the outer perimeters of the body cavity. Two distinct lobes were present in both anterior and posterior sections (Fig 4a,4b) separated by a thick central septum (Fig 5). The ventral surface of the gas bladder is located outside the visceral cavity and was attached to the parietal peritoneum (Fig 5).

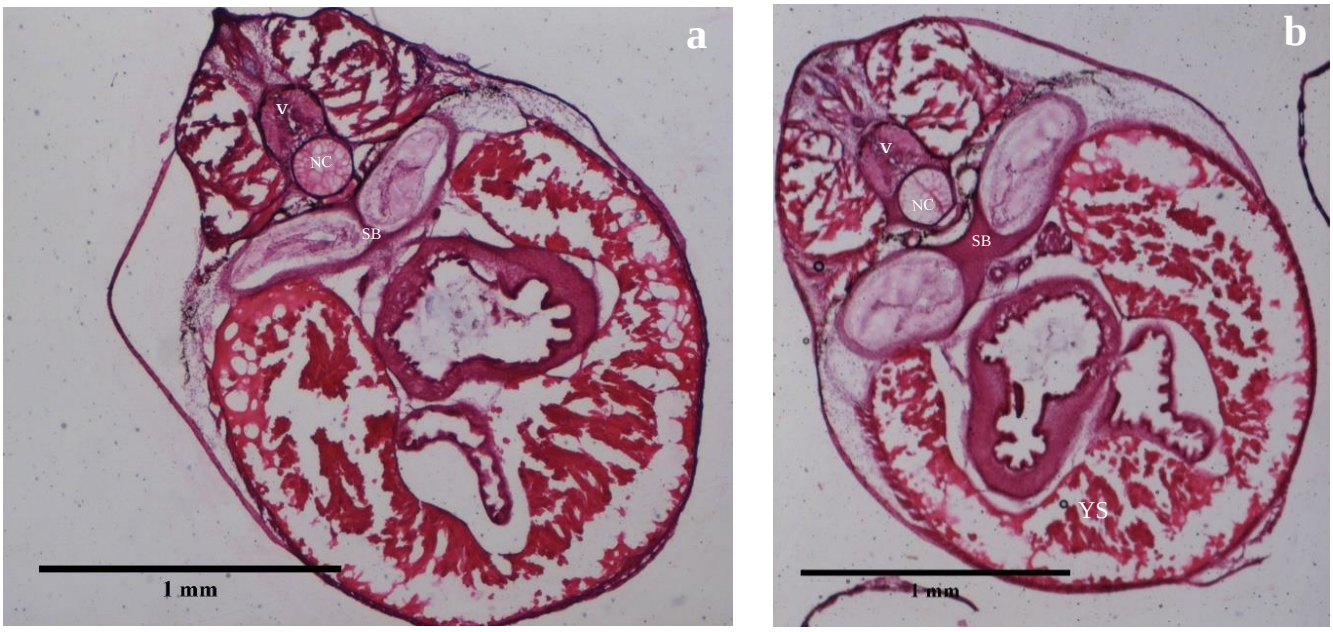

Figure 4: 30 µm thick transverse sections of *Ictalurus punctatus* larvae 8 days post-fertilization showing the internal structure including the vertebrae (V), notochord (NC), yolk sac (YS) and swimbladder, (SB). Void within the yolk was a result of damage during preparation of slides. Section (a) was directly posterior to section (b).

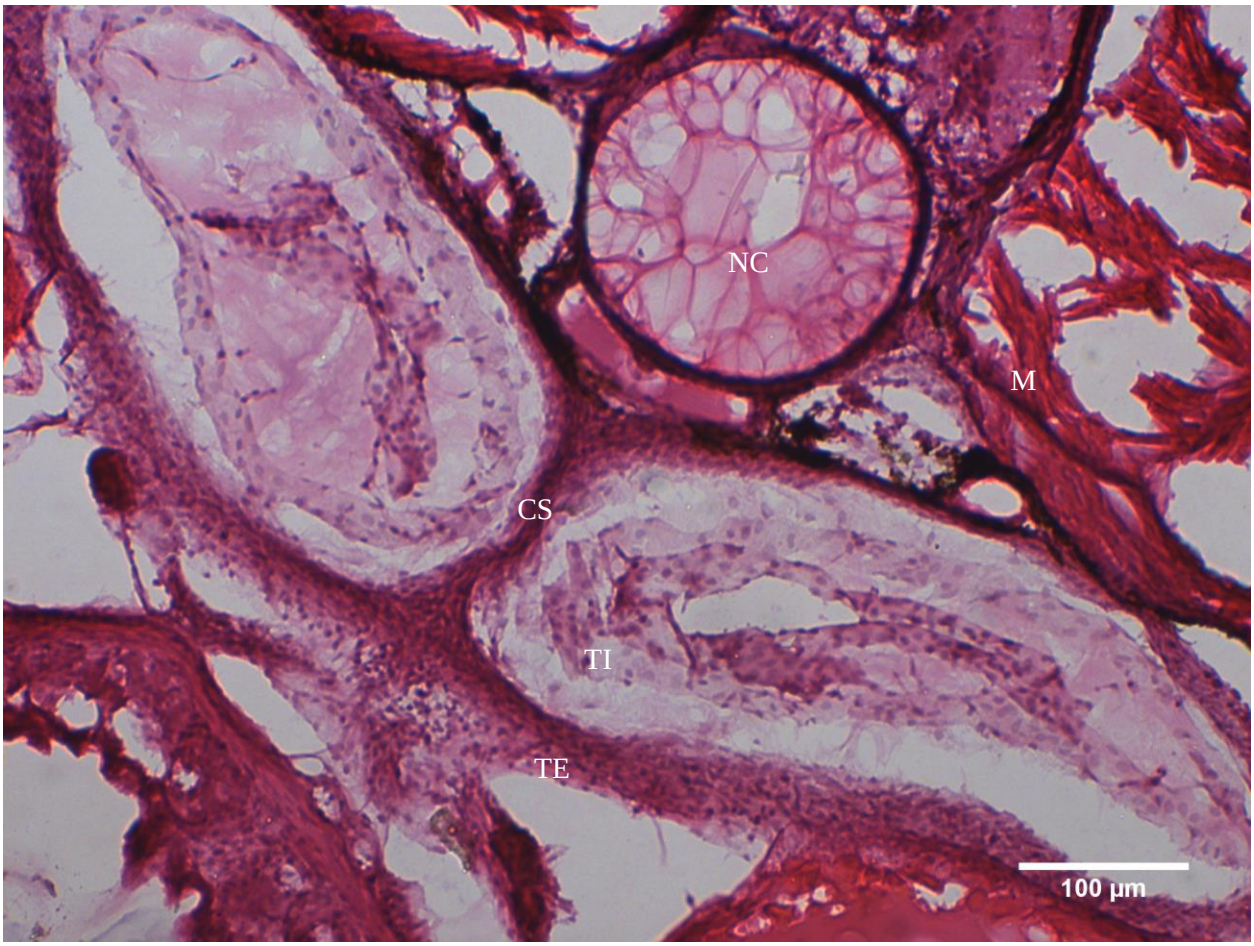

Figure 5: Expanded view of *I. punctatus* swimbladder at 9 days post-fertilization. The notochord (NC) was positioned directly above the swimbladder and was surrounded by skeletal musculature (M). The outer margin of the gas bladder or tunica externa (TE) was comprised of a layer of connective tissue and connects to the parietal peritoneum. Squamous epithelium lines the internal layer or tunica interna (TI).

***P. hypophthalmus* oxygen challenge**

Striped catfish larvae at 189 degree days had a survivability of 0% when oxygen was lowered to 0 ppm. The fish were initially swimming, but after 30 minutes the dissolved oxygen level dropped below 1 mg L^{-1} (Fig 6), and at that point many fish were on the surface swimming rapidly. After 45 minutes the dissolved oxygen level reached 0, and 40% of the fish were situated on the bottom of the tank (Fig 6). After 70 minutes, all the fish were dead.

Fish 216 degree days began the challenge actively swimming in the container. The oxygen concentration started at 4.6 mg L^{-1} (Fig 7). After 15 minutes the oxygen concentration had dropped rapidly to 0.9 mg L^{-1}, and many fish responded by swimming up to the surface. After 30 minutes, the fish were swimming very quickly at the surface, and the dissolved oxygen concentration fell to 0.6 mg L^{-1} (Fig 7). At 45 minutes, the dissolved oxygen levels decreased to 0 mg L^{-1} and 30% of the fish dropped to the bottom of the tank. After 75 minutes, the dissolved oxygen level remained at 0 mg L^{-1} and at 216 degree days there was a 0% survival rate for the *P. hypothalamus* larvae (Fig 7).

When the fish were 243 degree days, the challenge began with a dissolved oxygen level of 5.4 mg L^{-1}. Oxygen dropped to 1.6 mg L^{-1} then 0.8 mg L^{-1} at 15 and 30 minutes, respectively (Fig 8). Once the dissolved oxygen level dropped below 1.6 mg L^{-1} the fish were actively swimming at the surface. At 50 minutes, the dissolved oxygen level reached 0 mg L^{-1} (Fig 8). After 75 minutes, 27% of the fish died and sank to the bottom and 10 minutes later an additional 13% died. The challenge concluded at 120 minutes, and there was a 60 % survival rate for the *P. hypophthalmus* larvae at a dissolved oxygen reading of 0 mg L^{-1}.

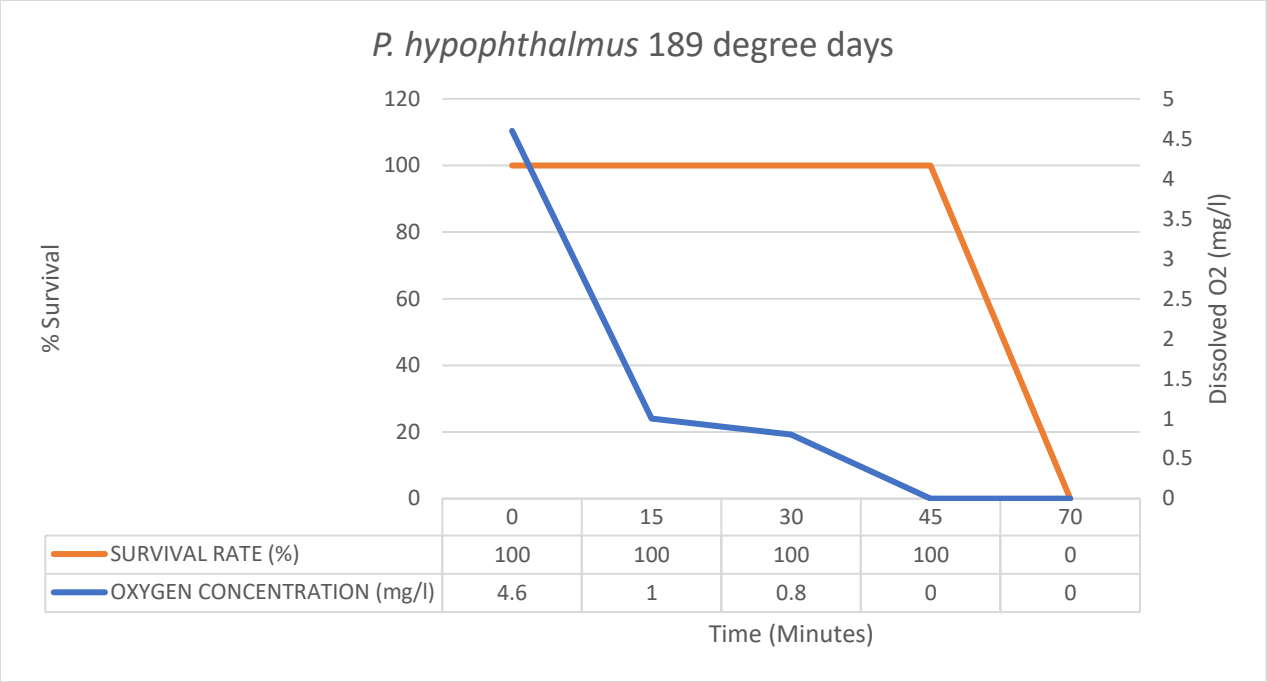

Figure 6: Dissolved oxygen levels (mg L^{-1}) and a survival curve for tra (*Pangasianodon hypophthalmus*) at 189 degree days for a low dissolved oxygen challenge. Dissolved oxygen level was decreased chemically with sodium nitrite. Catfish were determined moribund when forced ventilation of the opercula ceased.

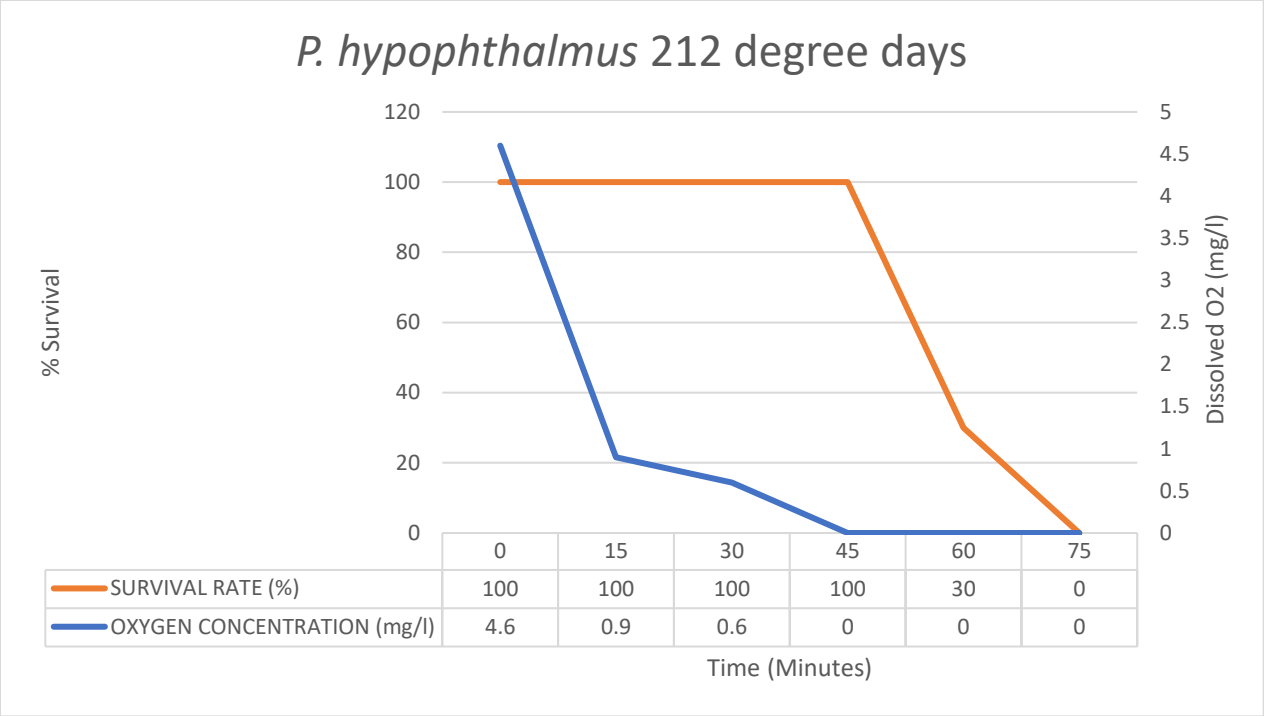

Figure 7: Dissolved oxygen levels (mg L^{-1}) and survival curve for tra (*Pangasianodon hypophthalmus*) at 212 degree days for a low dissolved oxygen challenge. Dissolved oxygen level was decreased chemically with sodium nitrite. Catfish were determined moribund when forced ventilation of the opercula ceased.

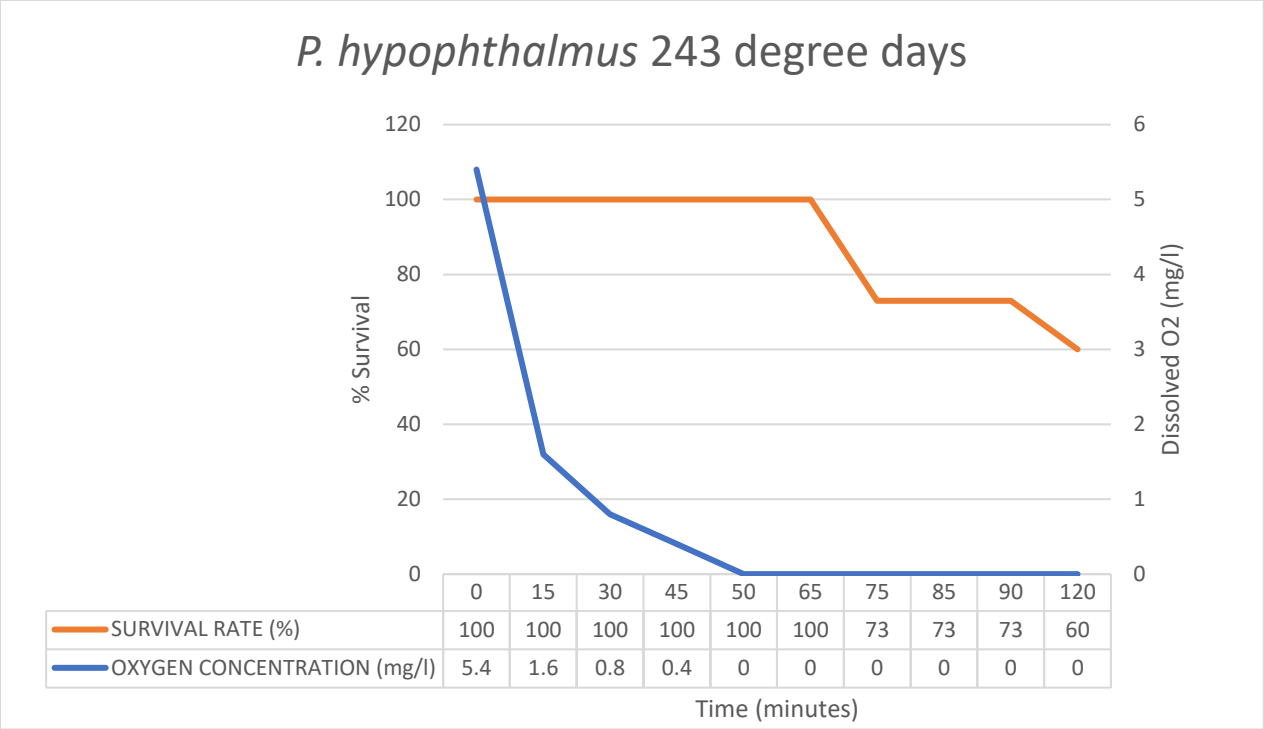

Figure 8: Dissolved oxygen levels (mg L^{-1}) and survival curve for tra (*Pangasianodon hypophthalmus*) at 243 degree days for a low dissolved oxygen challenge. Dissolved oxygen level was decreased chemically with sodium nitrite. Catfish were determined moribund when forced ventilation of the opercula ceased.

The challenge started at a dissolved oxygen reading of 4.6 mg L^{-1} for the tra larvae at 270 degree days, and the fish were actively swimming within the container. Thirty minutes in to the challenge, the dissolved oxygen reading was 0.5 mg L^{-1} (Fig 9). Fifty percent of the fish responded and were swimming at the surface. At 45 minutes the dissolved oxygen level fell to 0 mg L^{-1}, and all the fish were on the surface. At 60 minutes, 13% of the fish had died and ten minutes later an additional 7% had perished equating to 20% mortality (Fig 9). The challenge concluded after 120 minutes with the striped catfish larvae having 80% survival (Fig 9).

At 297 degree days the challenge began at 4.6 mg L^{-1} (Fig 10). The dissolved oxygen level was lowered to 1 and then 0.5 mg L^{-1} at 15 and 40 minutes, respectively (Fig 10). Fish were actively swimming at the bottom of the container. After 50 minutes, the dissolved oxygen level fell to 0 mg L^{-1} (Fig 10). At 0 mg L^{-1} dissolved oxygen, fish were swimming actively in the mid-water column with fish swimming to the surface to gulp air. The challenge was concluded after 120 minutes with 100% survivability of the *Pangasianodon* larvae (Fig 10).

Fish at 324 degree days had a similar result to the previous days larvae. The water challenge began with a dissolved oxygen level of 4.6 mg L^{-1} and was lowered to 0.9 mg L^{-1} after 15 minutes (Fig 10). Fish were actively swimming at the bottom of the container. After 40 minutes, the dissolved oxygen had reached 0 mg L^{-1} (Fig 10). There were no mortalities from that point until conclusion of the challenge. Fish were swimming actively making periodic trips to swim at the surface. The challenge was concluded after 180 minutes with 100% survival for the twelve-days post-fertilization larvae (Fig 10).

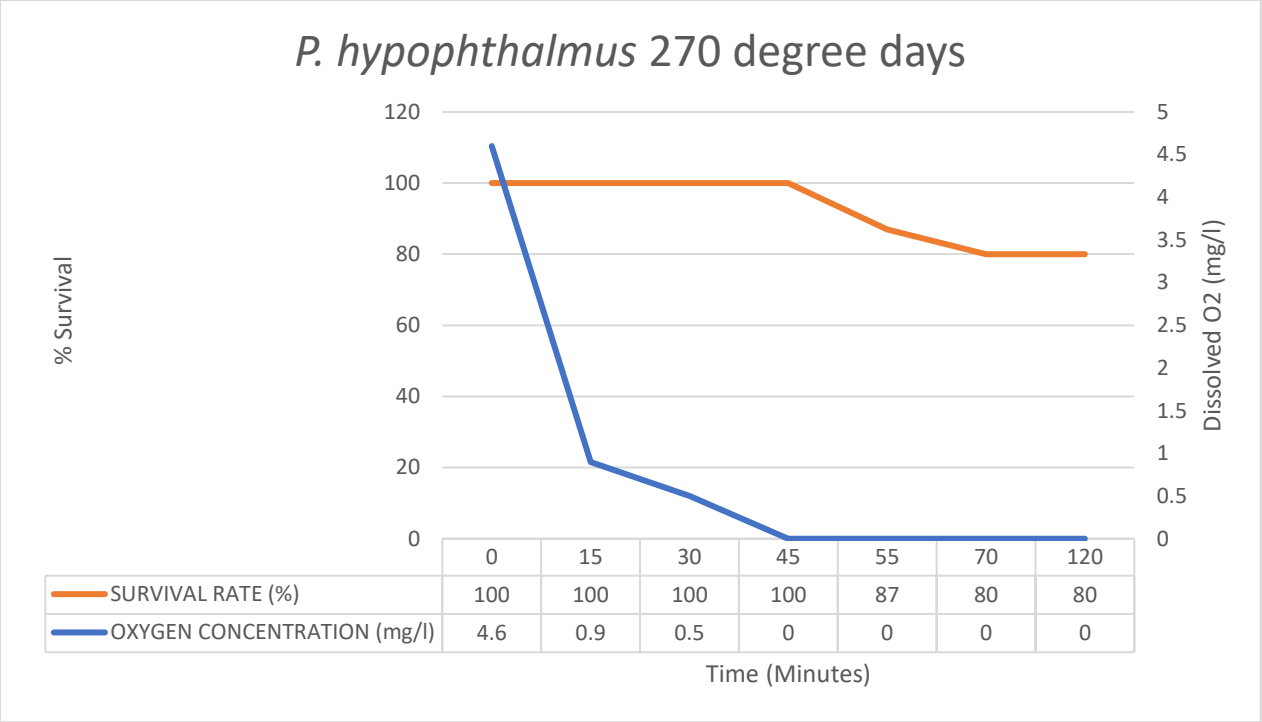

Figure 9: Dissolved oxygen levels (mg L^{-1}) and survival curve for tra (*Pangasianodon hypophthalmus*) at 270 degree days for a low dissolved oxygen challenge. Dissolved oxygen level was decreased chemically with sodium nitrite. Catfish were determined moribund when forced ventilation of the opercula ceased.

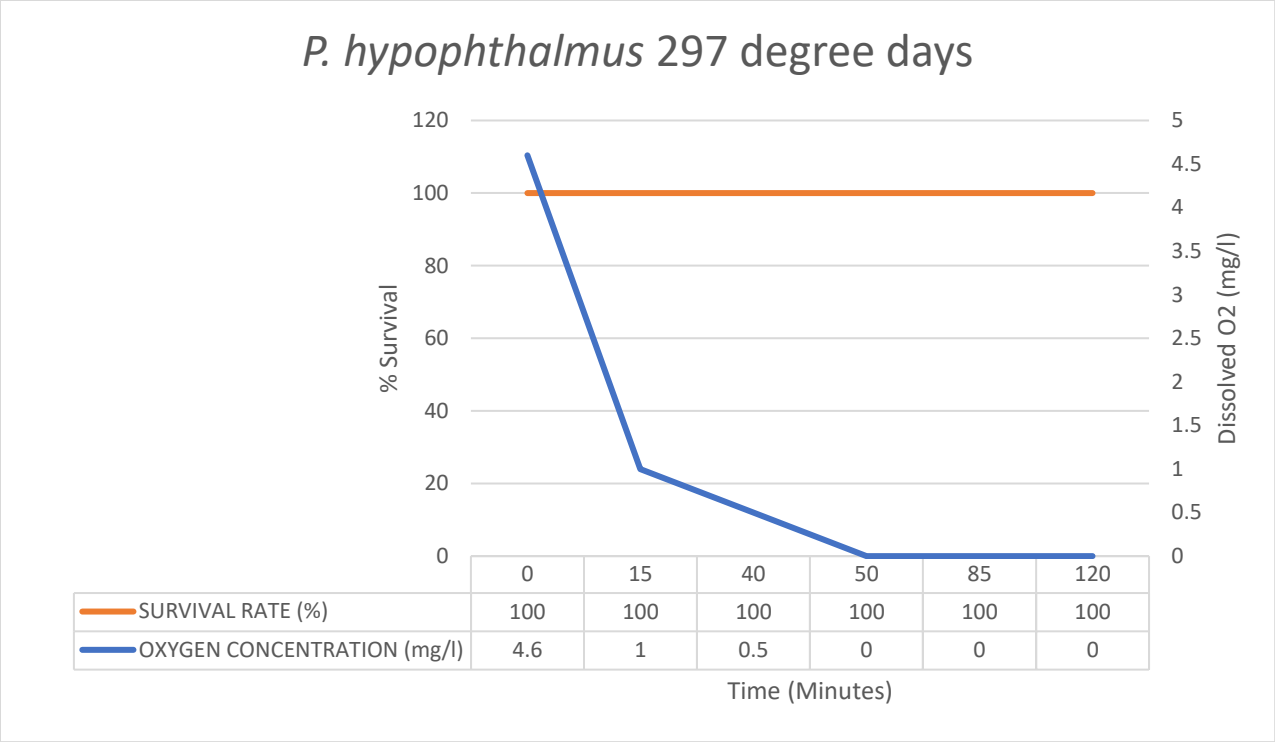

Figure 10: Dissolved oxygen levels (mg L^{-1}) and survival curve for tra (*Pangasianodon hypophthalmus*) at 297 degree days for a low dissolved oxygen challenge. Dissolved oxygen level was decreased chemically with sodium nitrite. Catfish were determined moribund when forced ventilation of the opercula ceased.

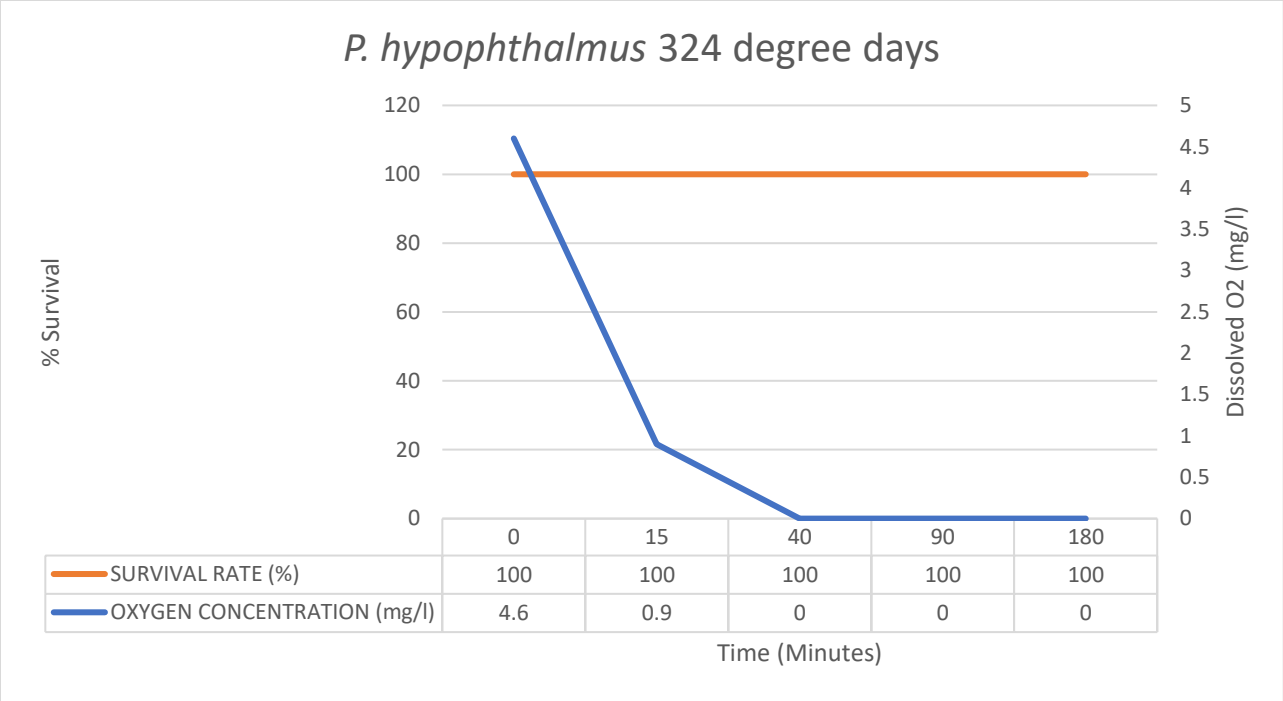

Figure 11: Dissolved oxygen levels (mg L^{-1}) and a survival curve for tra (*Pangasianodon hypophthalmus*) at 324 degree days for a low dissolved oxygen challenge. Dissolved oxygen level was decreased chemically with sodium nitrite. Catfish were determined moribund when forced ventilation of the opercula ceased.

Histology of the *Pangasianodon hypophthalmus* swimbladder

Pangasianodon hypothalamus larvae at 243 degree days were free swimming and could regulate their orientation in the water column. The yolk sac was absorbed entirely at this point, and the fry were reliant on exogenous feeding. Developed gastrointestinal structures were evident. Skeletal muscle surrounded the notochord and vertebral column. The swimbladder was situated retroperitoneally and encompassed a significant portion of the body cavity (Fig 12). Two chambers had been formed from the lumen and were divided by a longitudinal septum (Fig 12). The inner layer of the organ was comprised of cuboidal epithelium and the smooth outer layer was made up of elastic collagenic fibrosa (Fig 12). The tra larvae had a survival rate of 60% upon the conclusion of the dissolved oxygen challenge

At 270 degree days there were distinct changes to the morphology of the swimbladder. However, there was little change in the digestive tract in comparison to the previous sections. Musculature enveloping the vertebral column increased in thickness slightly. The swimbladder was expanded significantly with the inner layers pressed against the outer perimeter of the organ (Fig 13a,13b). The dimensions of the bi-lobed structure reached to the outer limits of the body wall with each lobed ovate in shape (Fig 13a,13b). No respiratory alveoli had developed, and the internal cavity of the organ was hollow (Fig 13a,13b). There was a small connection between the parietal peritoneum and the ventral portion of the swimbladder (Fig 13a,13b). The swimbladder was inflated immensely at this point and presumably became engorged with atmospheric air. An increase in survival rate occurred at this stage (Fig 9) equating to 80%.

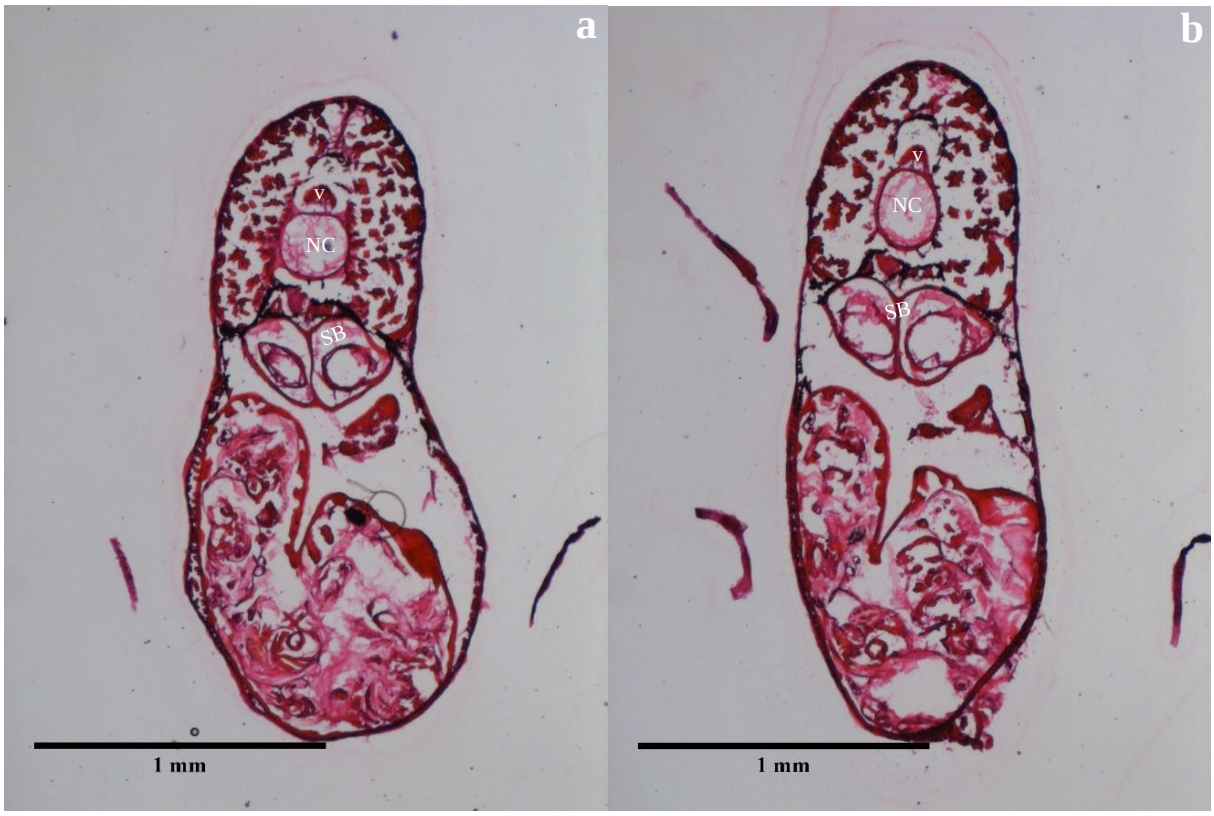

Figure 12: Transverse sections, 30 µm thick, from a *Pangasianodon hypophthalmus* 243 degree days. The vertebral column (V) was located just dorsal to the notochord (NC) and was surrounded by musculature. The developing swimbladder (SB) was distinctly bi-lobed and was located retroperitoneally.

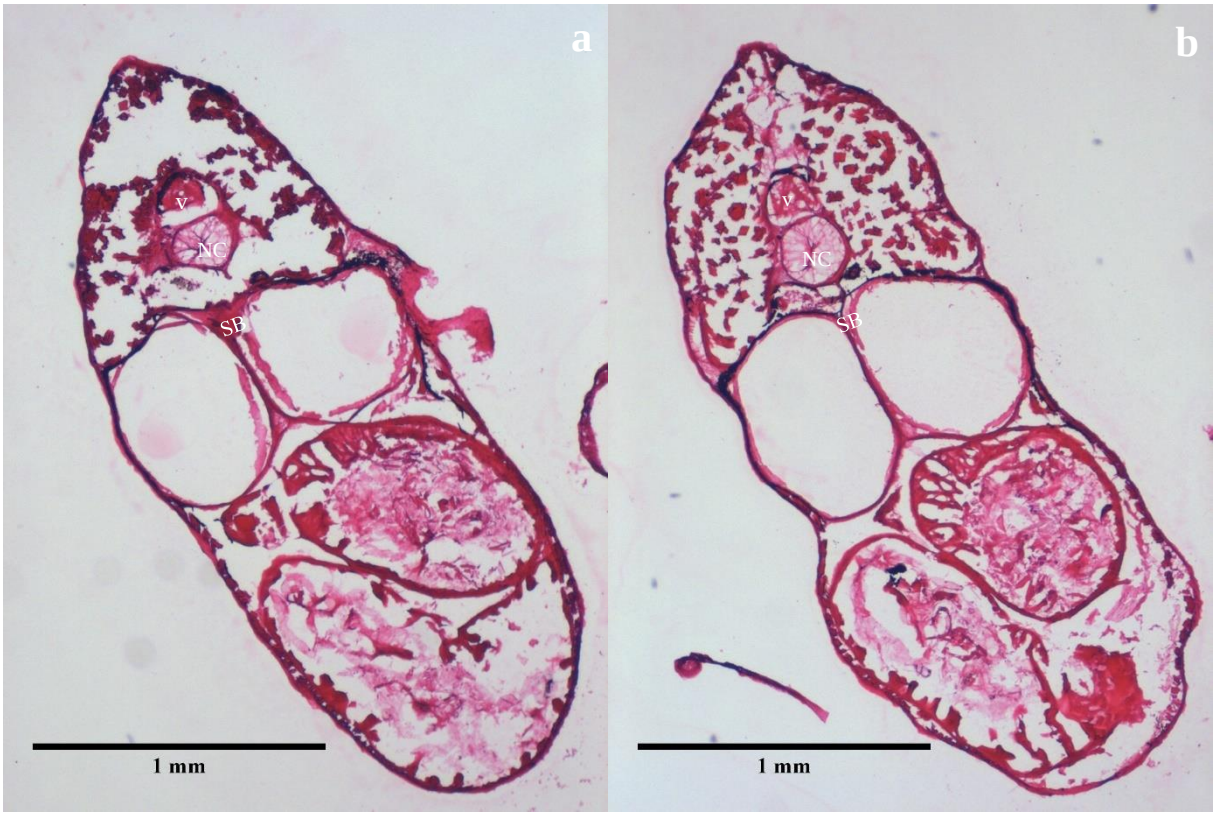

Figure 13: Transverse sections, 30 µm thick, from a *Pangasianodon hypophthalmus* at 270 degree days. The vertebral column (V) was located just dorsal to the notochord (NC) and was surrounded by musculature. The swimbladder (SB) was distinctly bi-lobed and was engorged to the perimeter of the body cavity.

Pangasianodon larvae at 297 degree days were similar in morphology to the sections of the previous day. Visceral structure was supported by mesentery within the peritoneal cavity. There was damage to the organs of the gut in the 297 degree days section making them appear hollow (Fig 14a,14b). There was a loose attachment to of the peritoneal cavity to the ventral portion of the swimbladder (Fig 14a and Fig 14b). The walls of the swimbladder had retracted slightly within the body and no longer extended to the distal portions of the body wall. The central septum was prominent in demarcating the parallel lobes (Fig 14b). Respiratory alveoli had not yet begun to invaginate from the internal layer of the ABO.

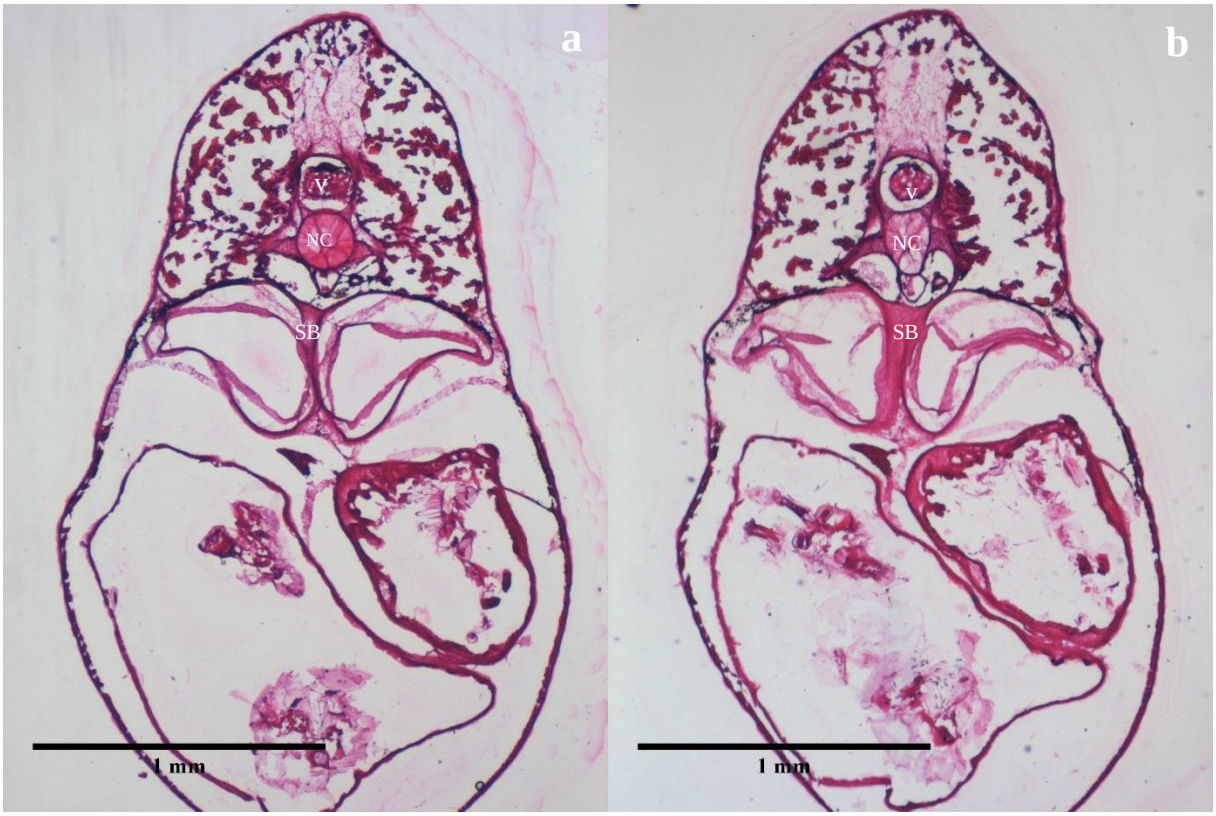

Figure 14: Transverse sections, 30 µm thick, from a *Pangasianodon hypophthalmus* at 297 degree days. The vertebral column (V) was just dorsal to the notochord (NC) and was surrounded by musculature. The swimbladder (SB) was distinctly bi-lobed and was located retroperitoneally.

Method development

High quality histological sections of catfish larvae were produced using the cryostat microtome. The highest quality cryotome sections were produced when the specimen was cut 12-72 hours after immersion in the 30% sucrose solution. Quality of the sample began to degrade if left in solution for more than 72 hours. The sucrose solution would soften the tissue causing damage during the freezing and cutting process and ending with poor quality sections. Modified regressive H&E staining techniques were able to display multiple cell types. Larval fish at different life stages required varying techniques to apply the correct amount of stain (Appendix A, Fig 1).

DISCUSSION

In this study, the development of respiratory structures in channel catfish (*Icatlurus punctatus*) and tra or striped catfish (*Pangasianodon hypophthalmus*) were compared histologically. Development of striped catfish was correlated with the onset of air breathing.

The functionality of the swimbladder as a regulator of buoyancy develops more quickly in *Pangasianodon hypopthalmus* than in *Ictalurus punctatus.* In this study, channel catfish at 185.5 degree days were confined to the bottom of the tank and were unable to maintain a position within the mid-water column. Observations of behavior and the results of the dissolved oxygen challenge at this stage show that the striped catfish at 189 degree days were significantly further along in development than channel catfish. This is not surprising as the time needed for them to hatch is also much less than in channel catfish. The swimmbladder was functioning as a buoyancy regulator as indicated by the tra swimming freely during the oxygen challenge. A 0% survival rate during the oxygen challenge indicated that the swimbladder had the capacity to uptake and store gases but did not function as an air breathing organ.

High water temperatures in tropical climates encourage development at a rapid rate such that key developmental stages overlap. The preflexion stage of larval development, defined by the complete digestion of the yolk sac and the start of flexion in the notochord coincided with the yolk sac stage of the newly hatched tra. A study by Morioka et al. (2009) showed that *P. hypophthalmus* larvae emerge from the egg with a yolk large yolk sac up to 36% of body length, but also started showing flexion of the notochord. Channel catfish emerge as yolk sac larvae and do not begin to show signs of flexion until further in development (265 degree days). The yolk sac was completely absorbed in *P. hypophthalmus* at 108 degree days and the larvae were free swimming. In this experiment, channel catfish relied on yolk sac for 291.5 degree days. There

was an aggregation of dark staining cells marking the location of the origin of the central septum at this stage. The absence of posterior parallel lobes indicated the gas bladder was still under development. The small cavities present within the organ had little capacity for the intake and storing of gases.

Channel catfish attained the ability to regulate their gas bladder and swim freely before the onset of air breathing in striped catfish. At 212 degree days, channel catfish showed an increase in diameter of both posterior and anterior regions of the gas bladder. In this study, the development of a thick central septum dividing the posterior section into two parallel chambers, and expansion of overall size directly coincided with the ability of the fish to remain buoyant within the water column. Morioka et al. (2009) and Islam (2005) showed that *P. hypopthalmus* had already achieved this as early as 112 degree days into development. *P. hypophthalmus* larvae were pelagically oriented at 206.5 degree days, and when exposed to hypoxic and subsequent anoxic conditions displayed 0% survival rate. A study by Zheng and Liu (1988) indicated that at this stage the pneumatic duct had not been formed and the pathway was not present to exchange gases within the swimbladder. The larvae facing hypoxic conditions in the challenge initiated air breathing behavior quickly upon sensing the decreasing dissolved oxygen level. Fish facing this stressor could be exhibiting the behavior because of instinct despite the lack of a pathway to the accessory respiratory structure. Alternatively, if the pneumatic duct is present the fish could have obtained the atmospheric air through buccal pumping forcing it into their swimbladder, but were incapable of processing the oxygen. The ability to regulate themselves in the water column would indicate that fish possess the ability to uptake and store air within their respiratory gas bladder. The pneumatic duct of the channel catfish was connected to both the swimbladder and

esophagus at 212 degree days and able to function as a regulatory device. The channel catfish were only limited in the amount of air uptake by the volume of their developing gas bladder.

Channel catfish larvae at 238.5 degree days had a swimbladder that was more fully developed than the previous day. The central septum was narrowed in comparison to the previous day. Al-Rawi (1967) observed a similar result. At 243 degree days, *Pangasianodon* larvae also showed a swimbladder divided into two posterior lobes with a much thinner central septum than their *Ictalurid* counterparts. The oxygen challenge data shows the onset of successful air breathing with a 60% survival rate.

The extension of the posterior lobes toward the body wall in both species at this point in development likely aids in enhanced auditory perception. The juxtaposition of the swimbladder to the body wall allows for the swimbladder to be used as a conduit for sound waves, directing them to the Weberian apparatus.

The development and functionality of gill structures was not compared in this study. High quality histological sections of the delicate gill lamelle were unable to be obtained for both species during initial stages of development. The earliest point in which histological sections were obtained for both species, 185.5 degree days for *I. punctatus* and 243 degree days for *P. hypophthalmus* showed remnants of damaged gill structures within the opercular cavity. The development of these primary respiratory structures were precursors to the development and functionality of the swimbladder as a regulator of buoyancy and as an accessory respiratory structure in channel catfish and tra, respectively.

In future studies, the respiratory structures of other air breathing fish can be analyzed histologically with slides cut on a cryostat microtome. The Thai walking catfish (*Clarias*

batarachus), another extensively cultured species in Southeast Asia that also have the ability to air-breathe. *Clarias spp.* species utilize an extension of the first gill arch in the suprabrachial chamber for gas exchange. Oxygen challenges during developmental stages correlated with histological analysis would determine critical time points for the functionality of this structure providing a comparison to striped catfish and channel catfish development.

Further investigation and experimentation utilizing RNA-Sequencing for transcriptome profiling would be beneficial for correlating gene expression to critical stages of development in gill, swimbladder, and accessory respiratory structures of these species. RNA sequencing data for channel catfish obtained at 132.5,185.5, and 238.5 degree days would show levels of expression at early, middle, and late stages of development of the swimbladder. *P. hypophthalmus* sampled at 189, 243, and 297 temperature days would reflect key developmental stages including, the absence of air breathing capability, onset of air breathing capability (60% survival), and a fully functional accessory respiratory structure. The thai walking catfish (*C. batrachus*) is an air breather not reliant on the swimbladder as an accessory respiratory structure. However, the air breathing organ sampled at time points before, during, and after the onset of air breathing behavior would be a comparative to the tra development.

Identifying the critical points in development can aid the advancement of best management practices for hatchery and grow out facilities within the aquaculture industry. Many farmers in Vietnam believe that tra obtain the ability to directly utilize atmospheric oxygen far later, around 30 days after hatch, than the results presented in this study (Dunham, personal communication). Surfacing behavior occurs in the striped catfish larvae at 243 degree days upon gaining the ability to obtain and process atmospheric oxygen. This energetically costly behavior

would make it beneficial for the culturist to maintain a dissolved oxygen level that discourages this action to allow the partitioning of resources for somatic growth rather than metabolism.

The development of air breathing structures of *Pangasianodon hypophthalmus* likely did not play a role in the development in the modern lung of vertebrates. A study by Brainerd (1994) concluded that the evolution of bimodal respiration in fish was associated with advancements in buccal pumping. The development of this system relied on the development of forced ventilation that can both intake and store air as well as expel it. Evolution of this bidirectional pump stemmed from the unidirectional pump used for suction feeding and gill ventilation in gnathostomes. Four-stroke ventilation is represented in all but two species of Actinopterygiians that use a primitive lung like a swimbladder as a gas exchanger with Sarcopterygiians utilizing a two-stroke pump (Brainerd 1994). The "four-stroke" method refers to the steps in air transfer. The buccal cavity expanded then constricted, drawing air from the lung to the buccal cavity which was then expelled. Then with a second expansion of the buccal cavity, fresh air was brought in from the surface, and with a subsequent compression of the cavity the lung was filled with fresh air through forced ventilation.

Browman and Kramer (1985) observed the surfacing behavior of adult *P. hypophthalmus* and noted that air was expelled from the opercular cavity not only when the fish returns from the surface, but also on the ascent. This behavior reflected the four-stroke system because of the release of spent air on the trip to the surface. Channel catfish also exhibit this behavior when piping or gulping at the surface when facing hypoxic conditions. Buccal compressions fill the swimbladder, but the lack of respiratory epithelium and the presence of guanine crystals on the inner lining of the swimbladder greatly diminish this capacity. Brainerd (1994) believed that these pumping systems developed independently within Actinopterygians given that the two-

stroke method the primitive form of ventilation in Sarcopterygians were more closely related to higher vertebrates. However, this cannot be proven without the presence of an air breathing out-group for comparison.

The utilization of a cryostat microtome to create high quality histological sections of larval catfish was accomplished in this study, but improvements in specific aspects of the method would broaden the scope for future research. The Harris Hematoxylin and Eosin staining protocol developed for this procedure can be improved upon to more accurately identify and enumerate specific cells and cell types. The staining process can be improved by fine tuning the exposure times of the slides to the primary stain and counter stain at distinct ranges in time of development for each species. The amount of dips and immersion time in the regression phase of the staining process would also change with different exposure levels to Harris Hematoxylin and Eosin. The addition of a bluing step using a saturated solution of lithium carbonate can often be incorporated into H&E staining protocols. This stain modifies the coloration of the chromatin within the nucleus to a blue/purple color enhancing contrast. A 0.3% hydrochloric acid alcohol solution applied after the primary stain could also be implemented to reduce background staining in histological sections. Determining the exact timing and position of these steps within the staining process would be subject to experimentation.

In conclusion, there were specific morphological changes that must occur for the swimbladders of channel catfish and striped catfish to function as a buoyancy regulator and accessory respiratory structure, respectively. These morphological changes were examined histologically using a cryostat microtome and a modified regressive Hematoxylin and Eosin stain. The development of the central septum was necessary before the ingestion of atmospheric air occurred through the pneumatic duct in channel catfish at 212 degree days, which coincided

with the swim-up stage. Comparatively, the onset of *P. hypophthalmus* successful air breathing occurred at 243 degree days despite the swimbladder already functioning as a buoyancy regulator at 112 degree days. Future studies will reveal more information regarding the development of air breathing behavior for socioeconomically important species.

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Appendix

Cryotome staining protocol

Materials:

1. Tissue sections mounted on 0.1% gelatin subbed slides
2. Electron Microscopy Sciences EasyDip slide staining system
    a. 12 staining jars
    b. 2 slide staining racks
3. Ultrapure or reverse osmosis H_2O
4. 95% and 100% ETOH
5. 10% Phosphate buffered formalin solution
6. Filtered Harris Hematoxylin
7. Eosin Y

Procedure:

1. Section larvae at 30 µm and mount on 0.1% gelatin subbed slides. Allow to air dry at room temperature for 30-60 minutes in a dust free environment.
2. To prepare the staining system:
    a. Prepare 1 jar of 10% phosphate buffered formalin, 3 jars ultrapure H_2O, 1 jar filtered Harris hematoxylin, 3 jars of 95% ETOH, 2 jars 100% ETOH, and 1 jar Eosin Y.
3. To clear Tissue Tek® and stain slides
    a. Place slide rack in 95% ETOH for 10-15 seconds
    b. Move slides to 10% phosphate buffered formalin for 10 seconds to fix samples.

c. Move slides into ultrapure H_2O and dip 4-5 times to remove Tissue Tek®.

d. For primary staining move slides to filtered Harris hematoxylin for 10-17 seconds depending on the age of the sample. Wipe off excess from slide rack with a paper towel.

e. Then dip the slides in ultrapure H_2O 3-5 times.

f. Move the slides to an immersion of ultrapure H_2O for 10 seconds.

g. To complete regression move the slides into a 95% ETOH solution for 10 seconds.

h. For counterstaining move slides to the Eosin Y for 10-15 seconds.

i. Move slides to a 95% ETOH solution and dip 3-5 times.

j. Transfer slide rack to a 95% ETOH for a 10 second immersion

k. Move the slides to a 100% ETOH bath for 5 seconds

l. Move the slides to a 100% ETOH bath for 10 seconds.