UNIVERSITY OF CALIFORNIA SAN DIEGO

Polypterus Breathing Physiology: An Investigation of Spiracle Use for Inhalation

A thesis submitted in partial satisfaction of the requirements for the degree in Master of Science

in

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by

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The Thesis of Lauren Ashley Miller is approved and it is acceptable in quality and form of publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Polypterus Breathing Physiology: An Investigation of Spiracle Use for Inhalation

by

Lauren Ashley Miller

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Professor David Woodruff, Chair

The role of spiracles in the respiration of *Polypterus*, a freshwater air-breathing fish, was investigated in order to clarify the long-standing debate of their use in aerial inspiration in this ancient lineage. Observations of four species (*Polypterus delhezi*, *Polypterus senegalus*, *Polypterus ornatipinnis*, and *Polypterus lapradei*) under conditions of varying temperature, oxygen and disturbance level provide insight into the frequency of air breaths and the breath cycle, along with conclusive evidence that *Polypterus* utilizes its spiracles for air-breathing. The level of disturbance was shown to have a direct impact on spiracle use; under "unstressed" or "natural" conditions spiracles were used preferentially for air inhalation. Under "stressed" conditions the mouth was the primary inhalation method. However, air-breathing frequency was found to be independent of

environmental factors including the temperature and dissolved oxygen content of the water, as well as disturbance levels. The breath cycle associated with *Polypterus* air-breathing was determined to have the following six key steps: 1) the head being brought parallel to the surface of the water, 2) the operculum expanding, 3) the floor of the mouth dropping with the release of the previous air breath, 4) the opening of the spiracular valve, 5) the floor of the mouth dropping a second time, and 6) the closing of the spiracular valve. Dissections performed as part of this research discovered a new muscle responsible for controlling the spiracular valve. The use of spiracles in *Polypterus* air-breathing may provide insight into the ecology and physiology of early swamp-dwelling tetrapodomorpha of the Devonian period.

Introduction

Purpose

This thesis reports a study of the breathing behavior and physiology of the African bichirs (*Polypterus)*, a diverse genus of fish having both gills and lungs, the latter being used for air-breathing. *Polypterus* also has two moderately large spiracles on its dorsal body surface just behind the eyes, and these have been postulated to have a role in air-breathing. The major focus of this work is to examine the role that these spiracles play in aerial respiration, and how their use is affected by environmental factors such as the oxygen content and temperature of the water, and behavioral stress.

Background

Polypterus is one of two genera in the family Polypteridae and of the order Polypteriformes. There are sixteen known species of *Polypterus*, all of which are bimodal breathers, meaning they can breathe both aquatically and aerially (Claeson et al, 2007). Also found in the family Polypteridae is the reedfish (ropefish) *Erpetoichthys calabaricus* (formerly in the monotypic genus *Calamoichthys*), a species that is also an air breather (Graham, 1997). Features of both of these genera include a thick covering of ganoid scales, fleshy, lobed pectoral fins, an asymmetric pair of lungs, four pairs of gill arches (most fish have four to five), and a pair of spiracles. Additionally, neither *Polypterus* nor *Erpetoichthys* are considered obligate air breathers, and earlier research shows that respiratory partitioning (i.e., the relative amounts of aerial and aquatic oxygen used in respiration) increases with growing body size and may differ among species (Graham, 1997, 2006).

Lungs

Polypterus has lungs. Although some early researchers termed this organ a swim or gas bladder, the lung is an entirely different organ. The fish gas (or swim) bladder has often been considered as homologous with lungs, but the two organs have a different development and morphology. Lungs develop from an outpocketing of the ventral wall of the alimentary canal that gives rise to a pair of ventrally aligned organs, while the gas bladder develops from an outpocketing of the side or dorsal part of the alimentary canal and leads to the development of a single organ, more dorsally located in the body than the lungs. Additionally, there is a valvular glottis located in the floor of the alimentary tract that guards the lung entrance; while there is no such valve found to guard the entrance to the gas bladder. Lungs also are connected to a pulmonary circulation, with vessels entering via a pulmonary artery and returning oxygenated blood to venous circulation returning to the heart. Gas bladders lack their own specialized blood vessels, and instead receive blood in parallel with the systemic circulation. It was thought that the lungs are the ancestral organs and that air bladders diverged from them (Graham, 1997). However as more recent research on actinopterygian lungs and gas bladders is conducted, more structural differences were found and the previous view that they came from a single origin is now widely disputed (Graham and Wegner, 2010). Perry (2007) further suggests that originally a posterior respiratory pharynx served as a rudimentary air-breathing organ, and from this organ lungs and the gas bladder likely developed in separate lineages.

Polypterus has two asymmetric sac-like lungs that are the same diameter, but the left lung is reduced in length to accommodate the stomach. Investigations of the

Polypterus lung by Lechleuthner et al. (1989) reveal that there are three functional layers A, B, and C, with A being the surface layer, and C being the innermost layer. Layer A is comprised of three types of cells: pneumocytes I, mucous cells, and pneumocytes II. Pneuomocytes I are flattened epithelium cells that are interconnected over the capillary bed and form the blood-air barrier. Pneumocytes II are composed of joined ciliated cells and lamellated epithelial cells. Layer B is made up of loosely arranged collagenous fibers of various thickness, elastic fibers, and vessels that supply and drain the lungs' capillary bed. Layer C is composed of muscle cells, including a sheet of smooth muscle and 2–3 sheets of striated muscle. Additionally, a peritoneal epithelium layer that acts as a membrane covers Layer C. *Polypterus* lungs also show specialized features, including the previously mentioned reduction of the left lung as well as a dorsal shift of both lungs that likely plays a role in achieving hydrostatic stability. The lung morphology of *Polypterus* and both the African and South American lungfish is functionally similar to that of primitive tetrapods and considered a model from which higher vertebrate lungs are derived.

Gills

Instead of the typical five pairs of gill arches found in primitive fish, *Polypterus* and *Erpetoichthys* only have four. Initially, there was a question of which gill arch was lost, the fourth or the fifth. An investigation by Britz and Johnson (2003) examined both possibilities, and ultimately determined that in *Polypterus* the last gill arch is the fourth arch and that the fifth arch is absent. Evidence for this conclusion includes several characteristics that are specific to the fourth gill arch of other actinopterygian fishes such as the presence of hemibranchs and blood vessels that are typical of the fourth gill arch.

Additional evidence is the absence of the fourth branchial trunk that typically innervates the fifth gill arch. Other fish exhibiting the loss of the fifth gill arch include some anguilliformes and clingfish, and chondrification of the fifth gill arch only occurs after arches 1–4 are formed. This collection of observations, led to the conclusion that the last arch of *Polypterus* is likely the original fourth gill arch, with some modifications, such as the muscles usually associated with the ancestral fifth gill arch, shifting to the last arch available.

Spiracles

Spiracles are paired vertical openings found in the majority of elasmobranchs, as well as several primitive bony fish, including those of the order Polyptiformes and the order Acipenseriformes (sturgeons and paddlefish), Lepisosteiformes (gars), and Amiiformes (bowfins) (Goodrich, 1958). These structures are considered remnants of the second gill slit that became reduced during jaw formation (Graham, 2006). In sharks and rays, spiracles are composed of either a pouch or tube, and covered by a stiff, crescent-shaped tissue fold, called the spiracular valve (Barry et al., 1987). This valve opens passively with current inflow, and closes by the action of the dorsal constrictor muscle. In this way, the valve acts in concert with fish oral breathing valves (Gudger, 1946).

Spiracle structure and function has been widely studied in sharks and rays, but less so in the primitive bony fishes. Burggren et al. (1985) investigated the breathing mechanism of sturgeon, which, like *Polypterus,* has spiracles. They found that when the mouth is buried on the ocean floor, the sturgeon (*Acipenser transmontanus*) inhales through a permanent opercular aperture that brings water to the gills. However, no evidence of water flow through the spiracles was seen in *A. transmontanus*, despite their

presence. Additionally, Magid (1966) postulated that water flows into the spiracles in *Polypterus,* though it is unknown if the role it plays is similar to the role of the opercular aperture in *Acipenser*.

The exact mechanism by which the spiracular valve opens is still unknown in *Polypterus*, but a study of its anatomy can give us some insight into possible mechanisms. *Polypterus* has two rows of irregularly shaped, bone-like plates (also referred to as spiracular ossicles and squamosoids) running along the top of the head, above the area covering the spiracle (Figure 1). Müller first described the valve like nature of its spiracle in 1843. Later, Traquair (1888) published an in depth examination of *Polypterus* cranial structure and these rows of plates. He described two small "spiracular ossicles" shaped like a triangle and a rectangle that formed a valve over the spiracular opening. Traquair also described four post-spiracular ossicles that ran behind the spiracular ossicles along the top of operculum arc, and two pre-spiracular plates that extended downward toward the frontal bone. He noted the irregularity of the shape and number of these structures, even stating that their number differed on the two sides of the head. However, he consistently reported two ossicles forming the valve that covered the spiracle. Nearly thirty years later, a description of these spiracle-covering structures was published by Allis (1908). He referred to them as ossicles and designated seven to eight pre-spiracular ossicles, two spiracular ossicles and three to five post-spiracular ossicles. Allis further noted that the number of ossicles varied and appeared to be correlated with body size (= number of finlets) and suggested that the number of ossicles, though seemingly irregular, was likely a specific character. More recently, Jollie (1988) examined the "spiracular ossicles" and renamed them squamosoids (accessory plates).

As did Traquair and Allis, Jollie observed the irregular nature of the lines of these plates with the exception of the two larger plates cover the spiracular opening. He also noted that these plates are a secondary layer overlying deeper, original bones, and that they may not be present in specimens smaller than 37 mm, but are well developed in larger specimens.

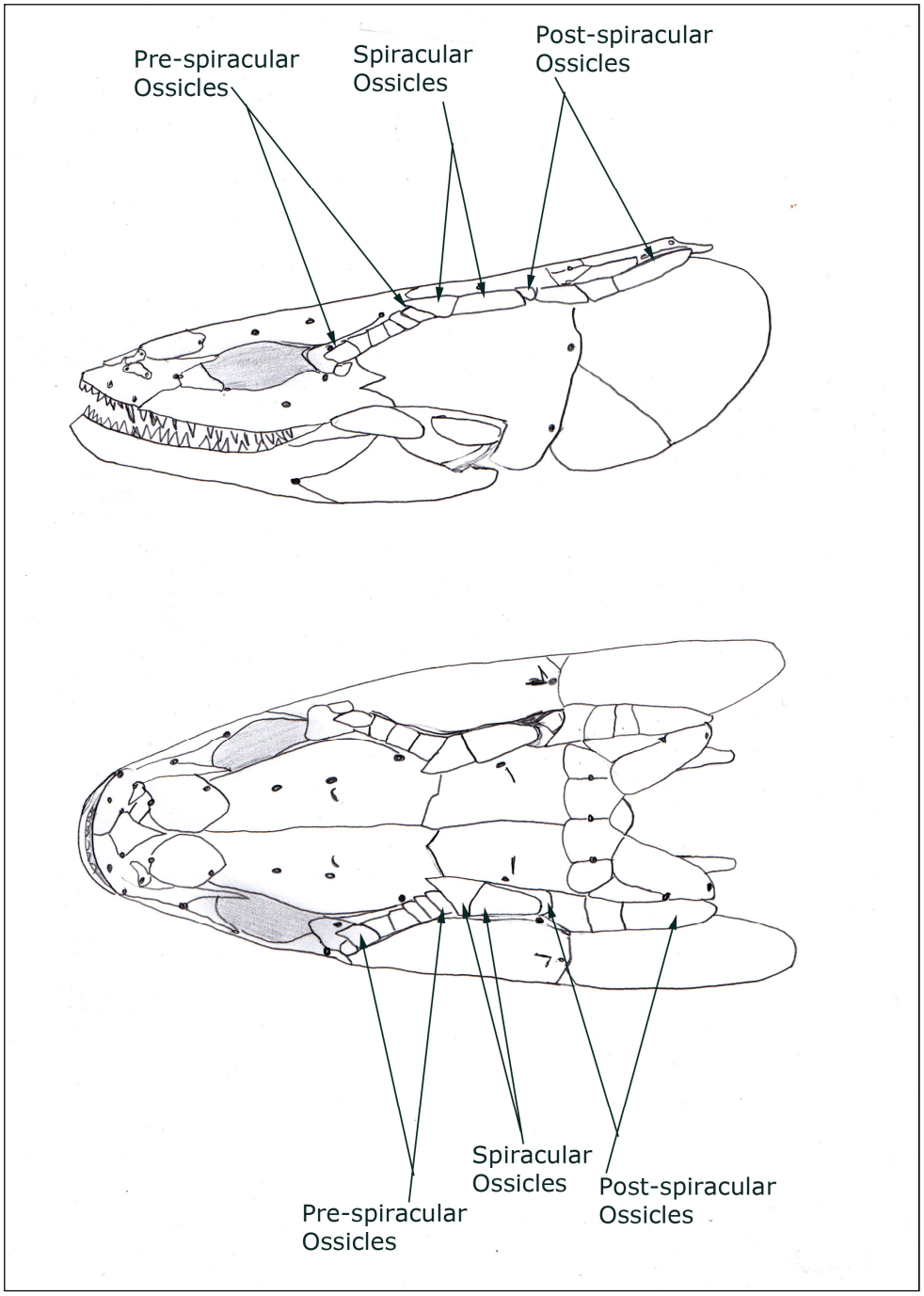

Figure 1: *Polypterus* cranial anatomy, including spiracular bones. Adapted from Allis, 1922.

The opening and closing of the spiracular valve described in *Polypterus* must be under the control of muscles. The muscle of interest in this regard is the *musculus*

spiracularis (Figure 2). The precise mechanism of its action is unknown. It was originally not recognized as an independent muscle, and was described by Pollard (1892) as a “slip of muscle” associated with the *dilatator operculi* and was inserted on the spiracular ossicles (squamosoids). The *musculus spiracularis* was first named and described as its own muscle by Luther (1913). He reported that the *musculus spiracularis* is a thinner layer of muscle that runs underneath spiracular plates and originates from the frontal bone. He also noted that the equivalent muscle in *Erpetoichthys* is reduced when compared to *Polypterus*. Allis (1922) described this muscle in more detail and explained that the *musculus spiracularis* is one of four muscles (the other beings the *levator arcus palatini* (*levator maxillae superioris* of Pollard), the *protractor hyomandibularis,* and the *dilatator operculi*) differentiated from one primitive muscle called the *levator arcus palatini*. In contrast with Pollard, Allis describes the *musculus spiracularis* as a wholly independent muscle that runs along the dorsal edge of the *dilatator operculi* (Figure 2). He describes the muscle as running just beneath the spiracular ossicles, posteriorly along the lateral edge of the spiracular opening and likely inserting on the spiracular opening. Like Luther, Allis states that the muscle originates on the hind edge of the frontal bone.

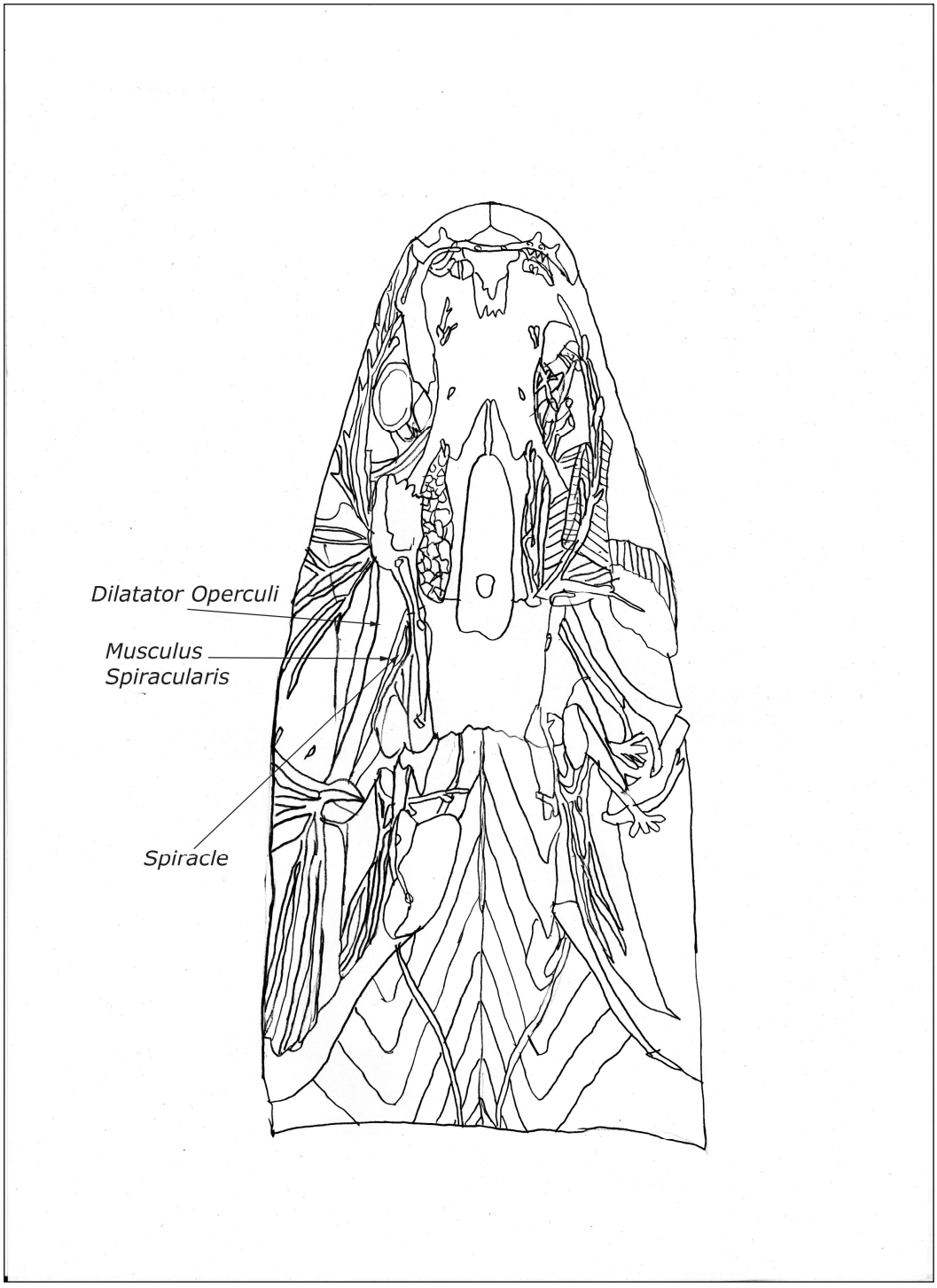

Figure 2: Muscles of the *Polypterus* head, including those most likely to be involved in spiracle opening and closing. Adapted from Allis, 1922.

Previous Research

John S. Budgett (1903) was the first scientist to study the use of the spiracle for air breathing in the early 1900s. He observed specimens of *Polypterus* that he kept in captivity and found that spiracles were used to inhale, as well as exhale air. During a one hour-long observation period of two fish, he counted a total of eight breaths and found that 50 percent of the time, the spiracles instead of the mouth were used to inhale air. Budgett concluded that *Polypterus* occasionally utilizes its spiracles to both inhale and exhale air from the "swim bladder" (which was the term used for the lung at that time). He also observed that in shallow water, *Polypterus* uses its spiracles preferentially compared to its mouth.

Over two decades later, Purser (1926) focused his research on *Erpetoichthys* respiratory and alimentary systems. He paid specific attention to the structure of the two lungs, the spiracles and operculae, and the thick muscular body. His observations of the respiratory system combined with Budgett's observations of *Polypterus* breathing behavior allowed him to speculate on a possible breathing strategy for *Polypteridae*, called recoil aspiration breathing. Instead of using a pump to force air into the lungs, *Polypterus* utilizes recoil aspiration, which involves the pulling of air into an expanding lung when the chamber is opened to the atmosphere. In other words, air is sucked into the lungs through the mouth instead of being pumped into them through an action similar to swallowing that occurs after the mouth has closed. In order to breathe in this way, *Polypterus* must generate negative pressure in its body cavity, and this is achieved by contraction of the lung walls and through the deformation of its stiff, heavily scaled body. Purser reasoned that exhalation occurred when the fish's body-wall contracted and

compressed the lungs. Relaxation of lung compression and expansion of the body wall caused inhalation. Purser also surmised that when the gill chamber expands the fish's mouth automatically closes, indicating that the spiracle is likely used in inhalation.

Research by Magid (1966) confirmed Budgett's conclusions about spiracle use in inhalation. It also agreed with Purser's conclusion that *Polypterus* did not use the typical buccal pump breathing method seen in other air-breathing fishes.

Over forty years after Purser proposed the recoil aspiration breathing method for *Erpetoicthys*, Brainerd et al. (1989) observed the same breathing method in *Polypterus*, emphasizing that it differed from the buccal pump, the air-breathing method of most other air-breathing fishes. They noted that the body is deformed dramatically during exhalation and returns to its original shape during inhalation as the lungs fill with air. However, they failed to cite Purser's research. They concluded that their discovery of recoil aspiration provided insight into the breathing techniques of early amphibians, that with their stiffly scaled bodies, may have also utilized aspiration breathing. Brainerd et al. also discussed the use of spiracles by *Polypterus*, and concluded, "they do not, as others have suggested, breathe through the spiracles." This conclusion is in direct opposition to previous research by Magid and Budgett. Additionally, it goes against current observations performed in the Graham laboratory.

Objectives

This study focuses on further examination of the role that spiracles have in air-breathing and the general breathing physiology of *Polypterus.* The objectives were to examine: 1) the role of spiracles in air-breathing, 2) the breath cycle, 3) the effect

different environmental conditions have on breathing behavior, and 4) the mechanism associated with spiracle opening and closing.

Materials and Methods

Experimental Subjects

Four *Polypterus* individuals of different species were observed to examine air-breathing physiology and behavior. Included in this study were single specimens of: *P. delhezi* (140.70 g, total length = 29.7 cm), *P. senegalus* (34.75 g, total length = 19.8 cm), *P. ornatipinnis* (134.23 g, total length = 29.4 cm), and *P. lapradei* (53.81 g, total length = 21.2 cm). The *P. delhezi* individual was blind, and this characteristic proved useful in the study of the effects of disturbance on air-breathing behavior. When not being observed, the fish were kept in clear water in separate tanks with heaters (24.5–26.0 °C) and filters. Their health was monitored daily and they were treated with antibiotics as needed. Fish were on a diet of live feeder goldfish, receiving 8–10, every 7–14 days. Each tank also contained a section of PVC pipe for the fish to hide in.

Experimental Set Up

The key research component revolved around observing and making video records of the natural air-breathing behavior of *Polypterus*. A series of 1–7 h observation periods were made, totaling over 325 h. Each fish was observed in a large aquarium (60.0 cm x 30.5 cm x 30.0 cm, l x w x d) that was partitioned to confine the fish in the front third of the tank (60.0 cm x 11.0 cm x 10.5 cm, l x w x d) while still allowing it to swim freely. During an observation period, the tank water level was brought to the very top of the tank in order to avoid visual parallax. Ambient light was reduced, except for spotlights directly over the tank, which enhanced video images. The observation room was kept quiet in order to minimize factors that might disrupt the fish's natural breathing pattern.

The video camera used was a Sony HDR–UX7, which allowed recording in both high definition and slow motion. Before the observation period began, the camera was set up and was positioned either to the side of the tank and level with the water surface, or above the tank and perpendicular to the water surface. This allowed documentation of both a side view and top view of the fish. It was important to document whether the mouth, the spiracles, or both were open at the time of inhalation. In videos taken from the side, the key objective was to record the timing of the elevation of the spiracular valve, above the water surface, which indicated the spiracles were open. Overhead videos similarly targeted the top of the head when it was at the surface with open spiracles.

Experimental Variables

The effects on air breaths of manipulation of water oxygen content and temperature were also observed. A YSI Model 58 dissolved oxygen meter was utilized to monitor both oxygen and temperature levels throughout the observation period (Table 1). Oxygen content was decreased by bubbling in nitrogen gas to displace oxygen in the water. The temperature of the water was increased by turning on additional heaters placed in the tank.

Table 1: Initial and final temperature and oxygen ranges for observation periods. (Dashes indicate that no data was obtained.)

	Temperature Levels (°C)		Oxygen Levels (mg/L)	
	Initial	Final	Initial	Final
P. delhezi, unstressed	25.1–25.7	25.1–25.7	—	—
P. senegalus, unstressed	26.2–26.6	27.1–31.5	2.7–5.9	0.6–1.4
P. senegalus, stressed	24.8–25.9	27.6–30.1	—	—
P. ornatipinnis, unstressed	24.0–28.6	29.3–32.8	0.9–7.1	0.0–2.2
P. ornatipinnis, stressed	24.9–24.8	28.6–32.6	—	—
P. lapradei, unstressed	25.4–28.3	29.0–31.5	1.1–4.7	0.2–1.2

Observations were refined to consider another variable, the disturbance level of the fish. This was done by comparing behaviors in “stressed” and “unstressed” fish. Only *P. senegalus* and *P. ornatipinnis* were observed under both conditions. Under stressed conditions, the observation tank was open to the surrounding laboratory, which meant the fish could experience ambient room conditions and movement of the researcher and other people in the room. For unstressed tests, a tent-like enclosure with a blind was set up around the tank and the fish was unable to see the researcher or the surrounding environment. Also, fake strips of polypropylene ribbon simulating natural foliage were added to the tank. This provided the fish with a place to hide. Ambient light in the enclosure was controlled by timers (0600 to 1800) to stimulate a “day” and “night” period for the fish. Additionally, pumps were set up to minimize the disturbance associated with changing the observation tank water level during observations and to maintain water circulation, which resulted in more uniform oxygen and temperature levels. The unstressed conditions were utilized for all four of the fish, including the blind *Polypterus delhezi*, which because it was blind potentially had an air-breathing behavior independent of visual stimuli associated with experimental setup. Additionally,

observations of two fish *(P. senegalus* and *P. ornatipinnis*) were performed in both stressed and unstressed conditions to compare surface breath frequency and spiracle use.

Video Editing and Analysis

During each observation period, the goal was to record individual breaths that the fish took and the percentage of these that involved inhalation via the spiracles or mouth and how this was affected during stressed vs. unstressed observation periods. Most of the videos were filmed in "slow motion" in which three s of "real time" was extended to 12 s of slow motion video. This slowed video, which could be slowed further by computer manipulation, enabled breaths to be examined in detail.

All video editing was done using Pinnacle Studio 12 Edition software, which allowed the video to be color corrected, slowed down, and spliced. Videos were analyzed to reveal the sequence of air-breathing events in exhalation and inhalation. Video analysis was also done to assess other breathing behavior activities, such as breathing frequency, the use of the mouth or spiracles, and the effects of stress vs. unstressed testing.

Statistics

Values of both spiracle use and air-breathing frequency were calculated and expressed as an average ($\bar{x}$) ± standard error (SE). For the Poincaré, linear regression lines were generated along with their associated coefficients of determination (R-squared values) using Microsoft Excel.

Spiracle Use

Breaths were categorized as spiracle breaths if both the spiracle was open, indicated by a visible spiracular valve in side view or open spiracle holes for top view,

and the mouth was closed and remained below the water surface. The total percentage of spiracle breaths observed was calculated by dividing the total number of spiracle breaths by the total number of breaths (spiracle breaths + mouth breaths). This analysis was carried out for each of the four species under unstressed conditions, as well as stressed and unstressed conditions for *P. senegalus* and *P. ornatipinnis.* The percentage of spiracle breaths per observation period was also calculated for every observation period. These percentages were then averaged for all of the observation periods that had the same fish and environmental condition to obtain six average percentages of spiracle use per observation period. These averages allowed one to compare the variation in the spiracle use for a fish from one observation period to the next.

Breathing Frequency

The frequency of air breaths was determined by calculating the average number of breaths per h in each observation period. This was calculated by first dividing the total number of breaths by the total number of h of an observation period to obtain a breathing frequency for each observation period. Next, all of these breathing rates for the same species and condition were averaged to obtain an average breathing rate per observation period for each fish and condition. Breathing frequency was also analyzed by looking at the interbreath interval (IBI). An IBI is the time between one breath and the subsequent breath. For example, the first IBI of an observation period would be the time between the first and the second breath observed. The average IBI for each fish under stressed and unstressed conditions (if applicable) was calculated. Additionally, Poincaré plots were generated for unstressed *P. ornatipinnis*, *P. senegalus*, and *P. lapradei*. *P. delhezi* was not included due to the limited oxygen and temperature data available for this fish. Poincaré

plots take a sequence of IBIs and plot each interval against the interval after it. A single point on the plot would consist of (IBI n, IBI n+1). They present the data in a way that may reveal self-similarity and the relationship between each interval to the next. If there is a relationship between subsequent breaths, such a plot forms a straight line, called the line of identity.

Breath Cycle

Breath cycles were examined for individual spiracle breaths of *P. delhezi* and *P. senegalus.* Key events that consistently occurred during every breath were identified and their timing was examined. Diagrams of the sequence of key events of the breath cycle were constructed for ten breaths of *P. delhezi* and four breaths of *P. senegalus*. To construct the activity series, the first key event was set at time zero, and the timing of each subsequent event was calculated from that point. Slow motion was converted to real time using a conversion factor of four.

Dissections

Five *Polypterus* specimens (spp.) were dissected to examine the muscles associated with air breathing and the opening and closing of the spiracular valve. Once the dissection technique was refined, two additional *Polypterus* specimens, identified as either *P. senegalus* or *P. cognicus,* were used. One had a total length of 20.0 cm and the other had a total length of 21.4 cm. These studies were performed under a dissection microscope and photographs were taken using a Canon Rebel camera. For each fish, the spiracular ossicles were removed and examined, looking for any muscles that made attachments directly to them. The number of pre-spiracular ossicles and post-spiracular ossicles were also counted and recorded for these two fish.

Results

Spiracle Use

Observation of four *Polypterus* species shows that spiracles are used for air inhalation, but the degree of spiracle use differs depending on environmental conditions and among the individual fish. The six combinations of species and experimental variables resulted in a range of spiracle breaths. Figure 3 and Table 2 show the total percentage of spiracle breath for the different species and experimental conditions. The range observed is from 8.47% (*P. ornatipinnis*, stressed) to 95.2% (*P. lapradei*, unstressed). Figure 4 and Table 3 show the average percentage of spiracle breaths per observation period for each species under stressed and unstressed conditions (when applicable). This also varies, ranging from 6.85 ± 3.50% (*P. ornatipinnis*, stressed) to 96.13 ± 2.85% (*P. lapradei*, unstressed). The percentage of overall spiracle breaths and spiracle breaths per observation period were dependent on whether the fish was in stressed or unstressed conditions. For all species observed, the total percentage of spiracle breaths in stressed conditions ranged from 8.47 to 53.44%, while the total percentage of spiracle breaths for unstressed conditions ranged from 76.79 to 95.74% (Figure 3). For the two species that were observed under both stressed and unstressed conditions (*P. senegalus* and *P. ornatipinnis*) there is a difference between the percentages of spiracle breaths, with the unstressed conditions have higher percentages (Figure 4). Table 3 shows the relatively large standard errors associated with all of the average percentages of spiracle breaths, emphasizing the variability of spiracle use during different observation periods.

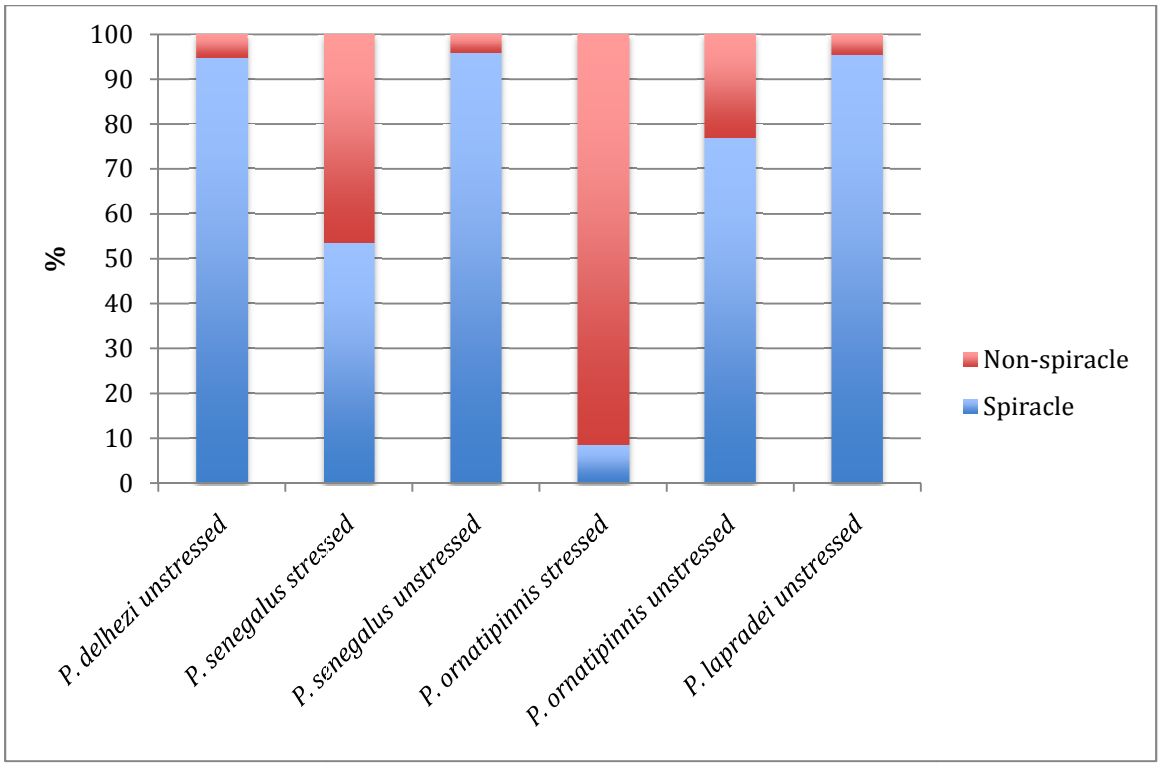

Figure 3: Relative frequency (%) of spiracle and non-spiracle air breaths observed for four species of *Polypterus* under different conditions (stressed and unstressed). Percentages were calculated from the following number of total breaths and observation periods: *P. delhezi* (167 breaths, 21 observation periods); *P. senegalus*, unstressed (235 breaths, 17 observation periods); *P. senegalus*, stressed (131 breaths, 17 observation periods); *P. ornatipinnis*, unstressed (56 breaths, 16 observation periods); *P. ornatipinnis*, stressed (59 breaths, 9 observation periods); *P. lapradei*, unstressed (84 breaths, 14 observation periods).

Table 2: Percentage of total spiracle breaths.

	% Spiracle Breaths
P. delhezi, unstressed	94.61
P. senegalus, stressed	53.44
P. senegalus, unstressed	95.74
P. ornatipinnis, stressed	8.47
P. ornatipinnis, unstressed	76.79
P. lapradei, unstressed	95.24

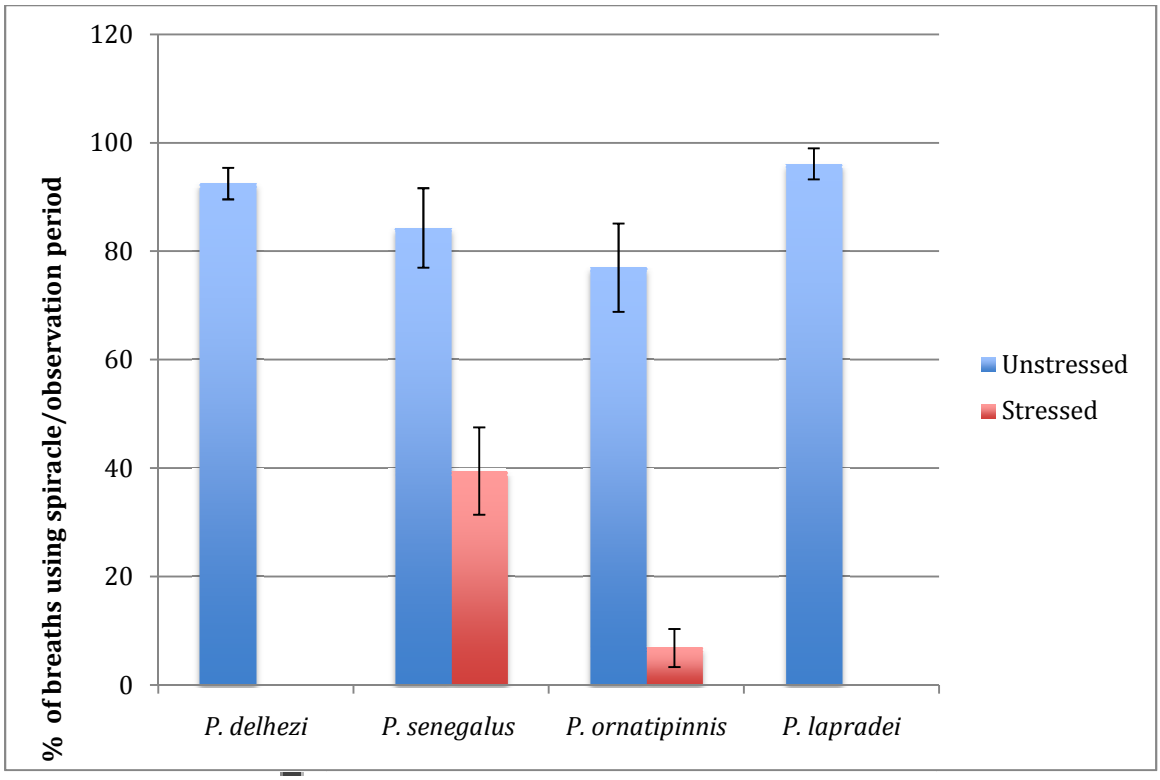

Figure 4: Average ($\bar{x}$ ± SE) percentage of spiracle breaths per observation period for each fish, under both stressed and unstressed conditions.

Table 3: Average ($\bar{x}$ ± SE) percentage of spiracle air breaths per observation period for all six fish and experimental conditions.

	% Spiracle breaths/observation period
P. delhezi, unstressed	92.50 ± 2.9171
P. senegalus, stressed	39.48 ± 8.0550
P. senegalus, unstressed	84.31 ± 7.3418
P. ornatipinnis, stressed	6.85 ± 3.4965
P. ornatipinnis, unstressed	76.99 ± 8.1359
P. lapradei, unstressed	96.13 ± 2.8463

Breathing Frequency

Breathing frequency was first analyzed by looking at the average number of breaths per h over each observation period for each of fish and the different experimental conditions (Figure 5). Frequencies ranged from 0.63 ± 0.16 breaths per h per observation period for *P. ornatipinnis*, unstressed to 12.48 ± 1.48 breaths per h per observation period

for *P. delhezi*, unstressed (Table 4). Although breathing frequency was expected to increase with decreasing levels of oxygen and higher temperature, this was not consistently seen. Also, there was no consistent pattern of differences in air-breathing frequency when comparing stressed and unstressed conditions for the two species examined under both conditions. For example, *P. senegalus* showed a higher air-breathing rate when unstressed, whereas *P. ornatipinnis* showed a higher air-breathing rate when stressed (Figure 5). Each fish studied had its own varying average breathing rate throughout an observation period, with unstressed *P. delhezi* having the highest (12.48 ± 1.48 air breaths per h per observation period) and unstressed *P. ornatipinnis* unstressed having the lowest breathing frequency (0.63 ± 0.16 air breaths per h per observation period) (Table 4). Each of the *Polypteru*s species also showed a relatively large variation of air-breathing frequency as noted by the values of their standard errors (Table 4). Individual observation periods were also expressed as the number of breaths per h in order to determine the effect of variables such as temperature and oxygen levels (Table 5–10). These tables allow for the examination of the change in breathing frequency throughout an observation period as water temperature and oxygen levels change.

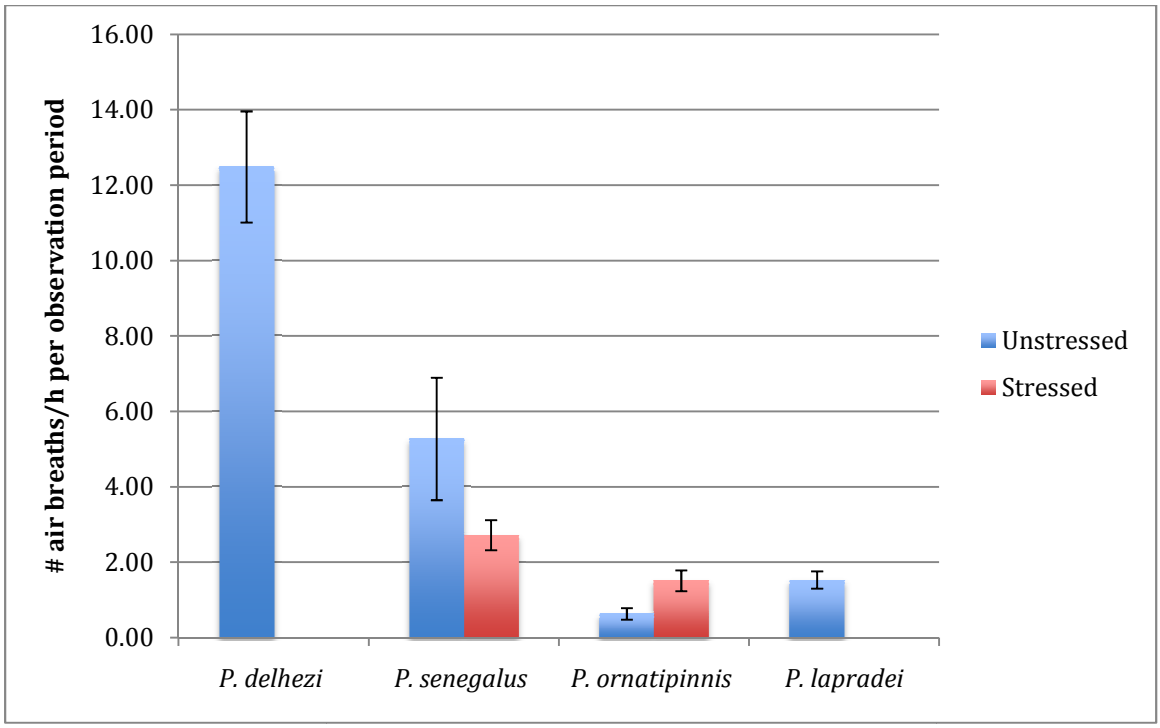

Figure 5: Average ($\bar{x}$ ± SE) number of spiracle breaths per h per observation period for each fish under both stressed and unstressed conditions.

Table 4: Average ($\bar{x}$ ± SE) number of spiracle breaths per h per observation period for all six fish and different experimental conditions. Also shows the total number of observation periods and h of observation.

	Average # breaths/h/observation period	Total # observation periods	Total # h observed
P. delhezi, unstressed	12.48 ± 1.48	37	61.15
P. senegalus, stressed	2.72 ± 0.40	17	62.92
P. senegalus, unstressed	5.27 ± 1.62	17	46.14
P. ornatipinnis, stressed	1.51 ± 0.27	16	77.06
P. ornatipinnis, unstressed	0.63 ± 0.16	9	39.00
P. lapradei, unstressed	1.53 ± 0.23	14	57.01

Table 5: *P. delhezi*, unstressed detailed breakdown of individual observation periods, higlighting the number of air breaths taken each hour. (Decimal in parentheses reflect fractions of hours.)

Date	Obs. Period (h)	mouth	spiracle	unknown	Total AB	h1	h2	h3	h4	Initial	Final	longest	shortest
9/17/08	1.08	0	11		11	10	1 (.08h)	---	---	24.3	---	11	1
9/19/08	2.23	0	21	5	26	14	9	3 (.23h)	---	25.1	---	11	1
9/23/08	1.32	0	8	0	8	6	2 (.32h)	---	---	24.9	---	24	4
9/30/08	2.32	0	10	0	10	3	4	3 (.32h)	---	24.8	---	32	5
10/3/08	0.12	0	0	2	2	2 (.12h)	---	---	---	24.9	---	---	---
	0.47	0	0	2	2	2 (.47h)	---	---	---	24.9	---	---	---
10/7/08	0.92	0	0	4	4	4 (.92h)	---	---	---	24.6	---	12	0
	1.48	0	0	28	28	20	8 (.48h)	---	---	24.6	---	10	1
10/9/08	0.28	0	0	3	3	3 (.28h)	---	---	---	25.2	---	5	4
	2.27	0	0	20d	20	9	9	2 (.27h)	---	25.2	---	13	2
10/10/08	3.93	0	0	68	68	14	15	16	23 (.93h)	25.0	---	10	1
10/14/08	1.42	0	0	14	14	10	4 (.42h)	---	---	24.8	---	16	1
	2.75	0	0	23	23	13	4	6 (.75h)	---	24.8	---	22	1
10/16/08	2.15	0	0	23	23	14	8	1 (.15h)	---	24.7	---	8	1
	0.35	0	0	6	6	6 (.35h)	---	---	---	24.7	---	13	1
	1.33	0	0	21	21	17	4 (.33h)	---	---	24.7	---	10	1
10/17/08	3.10	0	0	28	28	8	9	10	1 (.10h)	24.5	---	18	1
	1.00	0	0	26	26	26	---	---	---	24.5	---	8	1
10/23/08	1.72	0	0	30	30	20	10 (.72h)	---	---	25.1	---	9	0
	1.08	0	0	38	38	33	5 (.08h)	---	---	25.1	---	9	0
10/24/08	0.47	0	5	0	5	5 (.57h)	---	---	---	25.0	---	8	2
	1.18	0	14	0	14	13	1 (.18h)	---	---	25.0	---	12	1
10/30/08	0.63	0	4	0	4	4 (.63h)	---	---	---	24.6	---	12	2
	2.37	0	19	0	19	8	6	5 (.37h)	---	24.6	---	19	0
10/31/08	1.02	1	1	0	2	2	0 (.02h)	---	---	24.7	---	---	---
11/4/08	3.60	2	7	11	20	4	7	5	4 (.60h)	24.5	---	25	1
11/6/08	0.85	0	1	1	2	2 (.85h)	---	---	---	24.9	---	---	---
	2.92	1	14	1	16	4	8	4 (.92h)	---	24.9	---	29	4
11/7/08	0.92	0	1	0	1	1 (.92h)	---	---	---	25.0	---	---	---
	3.03	0	14	0	14	4	5	5 (.03h)	---	25.0	---	26	4
11/18/08	0.85	0	1	0	1	1 (.85h)	---	---	---	25.4	---	---	---
	1.62	1	4	1	6	4	2 (.62h)	---	---	25.4	---	28	10
3/30/08	1.73	0	3	0	3	1	2 (.73h)	---	---	25.4	---	26	16
4/1/09	3.00	1	9	0	10	3	3	3	---	25.7	---	26	6
4/2/09	2.28	1	4	0	5	2	3	0 (.28h)	---	25.1	---	30	23
4/6/09	1.28	0	2	1	3	2	0 (.28h)	---	---	25.3	---	39	14
4/7/09	2.08	2	5	0	7	3	5 (.08h)	---	---	25.4	---	41	2

Table 6: *P. senegalus*, unstressed detailed breakdown of individual observation periods, higlighting the number of air breaths taken each hour. (Decimal in parentheses reflect fractions of hours.)

		Air Breath Mechanics									Temperature (°C)		Oxygen (mg/L)		IBI (min)	
Date	Obs. Period (h)	mouth	spiracle	Total AB	h1	h2	h3	h4	h5	h6	Initial	Final	Initial	Final	longest	shortest
3/30/10	2.08	0	32	32	16	15	1 (.08h)	---	---	---	25.6	27.1	6.8	1.2	7	0
4/1/10	1.3	0	34	28	22	6 (.3h)	---	---	---	---	26.4	31.4	2.7	1	13	1
	4.17	0	37	37	7	11	11	13	3 (.17h)	---	26.4	31.4	2.7	1	12	1
4/5/10	4.23	0	20	20	4	5	5	6 (.23h)	---	---	26.5	30.6	4.8	1.1	23	2
4/7/10	3.13	0	14	14	3	6	5	---	---	---	26.2	29.1	3.2	1.4	22	2
4/8/10	2.25	0	16	16	9	7	0 (.25h)	---	---	---	26.6	31.5	3.3	0.6	16	1
	2.65	0	22	22	6	12	4	---	---	---	26.6	31.5	3.3	0.6	16	2
4/12/10	4.2	0	10	10	4	2	0	3	1 (.2h)	---	26.1	28.8	5.5	3.2	94	8
4/13/10	3.68	0	12	12	5	4	1	2	---	---	26.6	30.2	2.9	1.2	55	5
4/14/10	3.22	0	4	4	2	1	1	---	---	---	26.7	29.1	2.3	0.9	64	18
4/15/10	4.52	0	8	8	2	1	1	3	1 (.52 h)	---	26.3	30.5	3.1	1	56	9
5/4/10	4.3	0	5	5	0	0	1	3	1 (.3h)	---	26.8	31.4	4.6	0.8	67	12
5/5/10	3.88	1	4	5	1	1	1	1	---	---	27.4	31.2	0.9	0	63	23
5/6/10	4.1	3	2	5	1	0	1	3	0 (.1h)	---	29	32.1	2	0.4	74	23
5/13/10	5.22	1	4	5	0	1	2	1	1	0 (.22h)	27.1	31.4	4	0.6	81	32
5/19/10	5.62	3	0	3	0	0	1	1	1	0 (.62h)	26.8	31.1	4.3	1.4	86	62
5/24/10	4.37	2	1	3	0	0	1	1	1 (.37h)	---	27.2	31.1	5	0.6	56	38

Table 7: *P. senegalus*, stressed detailed breakdown of individual observation periods, higlighting the number of air breaths taken each hour. (Decimal in parentheses reflect fractions of hours.)

		Air Breath Mechanics									Temperature (°C)		IBI (min)	
Date	Obs. Period (h)	mouth	spiracle	unknown	Total AB	h1	h2	h3	h4	h5	Initial	Final	longest	shortest
4/8/09	2.62	4	0	0	4	2	0	1 (.62h)	---	---	25.7	---	53	42
4/14/09	2.57	3	3	1	7	4	3	0 (.57h)	---	---	25.9	---	23	5
4/15/09	3.57	7	2	1	10	2	3	3	2 (.57h)	---	25.4	---	33	12
4/16/09	2.2	2	0	0	2	1	1	0 (.2h)	---	---	25.1	---	---	---
4/21/09	1.48	2	0	0	2	1	1 (.48h)	---	---	---	25.9	---	---	---
4/22/09	3.7	6	4	0	10	3	2	3	2 (.70h)	---	25.2	---	31	13
4/23/09	2.3	2	0	0	2	0	2	0 (.30h)	---	---	25.4	---	---	---
5/11/09	2.02	1	0	0	1	1	0	0 (.02h)	---	---	25.6	---	---	---
5/12/09	2.67	1	3	0	4	1	2	1 (.67h)	---	---	24.8	29.2	46	19
5/13/09	4.03	4	3	0	7	2	1	2	2	0 (.02h)	25.0	30.0	74	17
5/18/09	1.92	2	2	0	4	2	2 (.92h)	---	---	---	25.3	27.7	37	25
5/19/09	1.45	2	3	0	5	4	1 (.45h)	---	---	---	25.0	28.0	30	10
5/20/09	3.95	6	11	0	17	3	3	5	6 (.95h)	---	25.3	30.1	27	5
5/26/09	1.8	3	4	0	7	5	2 (.80h)	---	---	---	25.5	27.7	48	1
5/27/09	4.03	2	19	0	21	4	5	10	2	0 (.03h)	25.2	29.5	28	2
6/2/09	2.15	0	13	0	13	8	4	1 (.15h)	---	---	25.3	27.6	29	2
6/3/09	3.68	13	3	1	17	3	4	6	4 (.68h)	---	25.6	29.9	33	5

Table 8: *P. ornatipinnis,* unstressed detailed breakdown of individual observation periods, higlighting the number of air breaths taken each hour. (Decimal in parentheses reflect fractions of hours.)

		Air Breath Mechanics		AB Physiology									Temperature (°C)		Oxygen (mg/L)		IBI (min)	
Date	Obs. Period (h)	mouth	spiracle	Total AB	h1	h2	h3	h4	h5	h6	h7	h8	Initial	Final	Initial	Final	longest	shortest
1/19/10	4.95	0	1	1	0	0	0	0	1 (.95h)	---	---	---	26.2	30.6	---	---	---	---
1/20/10	4.77	0	1	1	0	0	0	1	0 (.77h)	---	---	---	24.6	29.2	---	---	---	---
1/21/10	6.2	0	5	5	0	0	0	1	2	2	0 (.2h)	---	24	30.6	---	---	74	3
1/25/10	7.65	4	14	19	0	6	2	2	1	2	4	2 (.65h)	23.8	32.4	---	---	62	1
1/26/10	2.55	0	1	1	0	0	1 (.55h)	---	---	---	---	---	25.4	29.4	---	---	---	---
1/27/10	5.25	0	0	0	0	0	0	0	0	0 (.25h)	---	---	24.9	32	---	---	---	---
2/8/10	5.87	0	1	1	0	0	0	0	0	1 (.87h)	---	---	24.6	32.8	---	1.8	---	---
2/9/10	4.48	1	1	2	0	0	0	1	1 (.48h)	---	---	---	25.4	31.3	7.1	1.5	---	---
2/22/10	2.05	0	0	0	0	0	0 (.05h)	---	---	---	---	---	28.6	29.3	1.9	0.7	---	---
2/23/10	3.63	1	0	1	0	0	0	1 (.63h)	---	---	---	---	25.7	30.2	7	0.9	---	---
2/24/10	5.93	1	7	8	0	1	1	2	2	2 (.93h)	---	---	26.2	30.6	2.6	0.5	55	20
2/25/10	3.68	1	2	4	1	1	1	1 (.68h)	---	---	---	---	26.8	30.6	2.9	0.3	64	33
3/1/10	4.78	0	3	3	0	0	1	1	2 (.78h)	---	---	---	26.5	30.8	4.9	0.7	51	46
3/8/10	6.42	2	2	4	0	0	1	1	2	0	0 (.42h)	---	27.4	31.2	4.4	0.5	58	28
3/10/10	5.25	1	2	3	0	0	0	1	1	1 (.25h)	---	---	26.5	30.7	2.8	0.5	61	56
3/11/10	3.6	0	3	3	0	0	1	2 (.60h)	---	---	---	---	26.1	30.2	3.1	0.3	61	27

Table 9: *P. ornatipinnis,* stressed detailed breakdown of individual observation periods, higlighting the number of air breaths taken each hour. (Decimal in parentheses reflect fractions of hours.)

		Air Breath Mechanics											Temperature (°C)		IBI (min)	
Date	Obs. Period (h)	mouth	spiracle	Total AB	h1	h2	h3	h4	h5	h6	h7	h8	Initial	Final	longest	shortest
12/7/09	4.5	4	1	5	0	0	1	3	1 (.5h)	---	---	---	26	29.1	74	17
12/8/09	3.47	9	3	12	3	4	3	2 (.47h)	---	---	---	---	29.1	30.7	38	6
12/9/09	4.05	7	0	7	1	1	2	2	1 (.05h)	---	---	---	24.7	28.8	88	18
12/16/09	4.28	5	1	6	0	1	1	3	1 (.28h)	---	---	---	24.9	29.3	66	20
1/4/10	7.53	11	0	11	0	1	2	1	2	2	2	1 (.53h)	25.4	31.9	56	15
1/5/10	3.95	2	0	2	0	0	1	1 (.95h)	---	---	---	---	26.5	30.3	---	---
1/6/10	6.02	10	0	10	0	1	2	2	3	2	0 (.02h)	---	25.3	32.6	42	10
1/12/10	3.08	4	0	4	1	1	1	1 (.08h)	---	---	---	---	25	25	64	34
1/18/10	2.12	2	0	2	1	0	1 (.12h)	---	---	---	---	---	24.4	27	---	---

Table 10: *P. lapradei*, unstressed detailed breakdown of individual observation periods, higlighting the number of air breaths taken each hour. (Decimal in parentheses reflect fractions of hours.)

		Air Breath Mechanics										Temperature (°C)		Oxygen (mg/L)		IBI (min)	
Date	Obs. Period (h)	mouth	spiracle	Total AB	h1	h2	h3	h4	h5	h6	h7	Initial	Final	Initial	Final	longest	shortest
6/3/10	2.83	0	6	6	1	2	3 (.83h)	---	---	---	---	27.4	30.8	2.6	0.2	39	18
6/9/10	2.57	0	3	3	1	1	1 (.57h)	---	---	---	---	26.2	29	4.3	0.7	60	55
6/14/10	3.83	0	3	3	1	1	0	1 (.83h)	---	---	---	25.8	30.4	4.7	1.2	128	29
6/15/10	4.62	0	6	6	1	2	0	2	1 (.62h)	---	---	25.9	30.7	3.6	0.6	84	24
6/16/10	4.78	3	5	8	0	1	2	4	1 (.78h)	---	---	27.9	30.5	2.4	0.3	55	0
6/21/10	3.58	0	4	4	1	2	1	0 (.58h)	---	---	---	25.4	30.4	3.6	0.3	42	32
6/22/10	4.95	0	5	5	2	0	0	1	2 (.95h)	---	---	26.6	30.5	2.4	0.4	164	10
6/23/10	4.3	1	5	6	1	2	2	1	0 (.30h)	---	---	26.2	29.2	---	---	74	14
6/28/10	2.55	0	4	4	0	2	2 (.55h)	---	---	---	---	26.9	30.7	4.2	0.4	34	26
7/6/10	6.1	0	4	4	1	0	1	1	0	1	0 (.10h)	28.3	31.2	1.1	0.2	145	98
7/7/10	4.43	0	4	4	0		1	2	0 (.43h)	---	---	27.7	30.8	4.1	0.3	162	38
7/8/10	3.68	0	15	15	4	5	4	2 (.68h)	---	---	---	26.5	29.8	1.5	0.5	21	7
7/13/10	4.27	0	7	7	0	2	2	3	0 (.27h)	---	---	26.3	31.13	4.4	0.2	43	25
7/14/10	4.52	0	9	9	1	2	2	3	1 (.52h)	---	---	27.2	31.5	2.8	0.2	47	13

The combined IBI values shown in Tables 5–10 are summarized in Table 11 and Figure 6. These average IBI values ranged from 6.0 ± 0.00021 min (*P. delhezi*, unstressed) to 37.0min ± 0.0029 min (*P. lapradei*, unstressed) (Table 11 and Figure 6). There was no consistent difference seen between stressed and unstressed conditions when looking at the same fish, though IBI averages differed from one species to another. The identity lines generated via Poincaré plots for unstressed *P. ornatipinnis*, *P. senegalus,* and *P. lapradei* are highly variable for the single observation periods (Figures 7–9) and all except one show no correlation between IBI n and IBI n+1 (Figures 7–9). The one observation period that showed a relatively strong correlation, was for *P. ornatipinnis* on 2/24/10. This observation period had a line of identity of y = 0.5851x + 9.1496 and an R-squared value of 0.64934 (Figure 8).

Poincaré plots that lumped all of the IBIs for these three fish and condition produced lines of identity that showed a relationship between one IBI and the subsequent IBI for both *P. senegalus* and *P. ornatipinnis (*Figures 10–11). *P. senegalus* has a line of identity of y = 0.5843x + 4.7008 and an R-squared value of 0.3234, and *P. ornatipinnis* has a line of identity of y = 0.4393x + 14.263 and an R-squared value of 0.4138. On the other hand, a Poincaré plot constructed by lumping all IBIs for unstressed *P. lapradei* showed no correlation between IBI n and IBI n+1, indicated by its relatively low R-squared value (R-squared = 0.12374) (Figure 12).

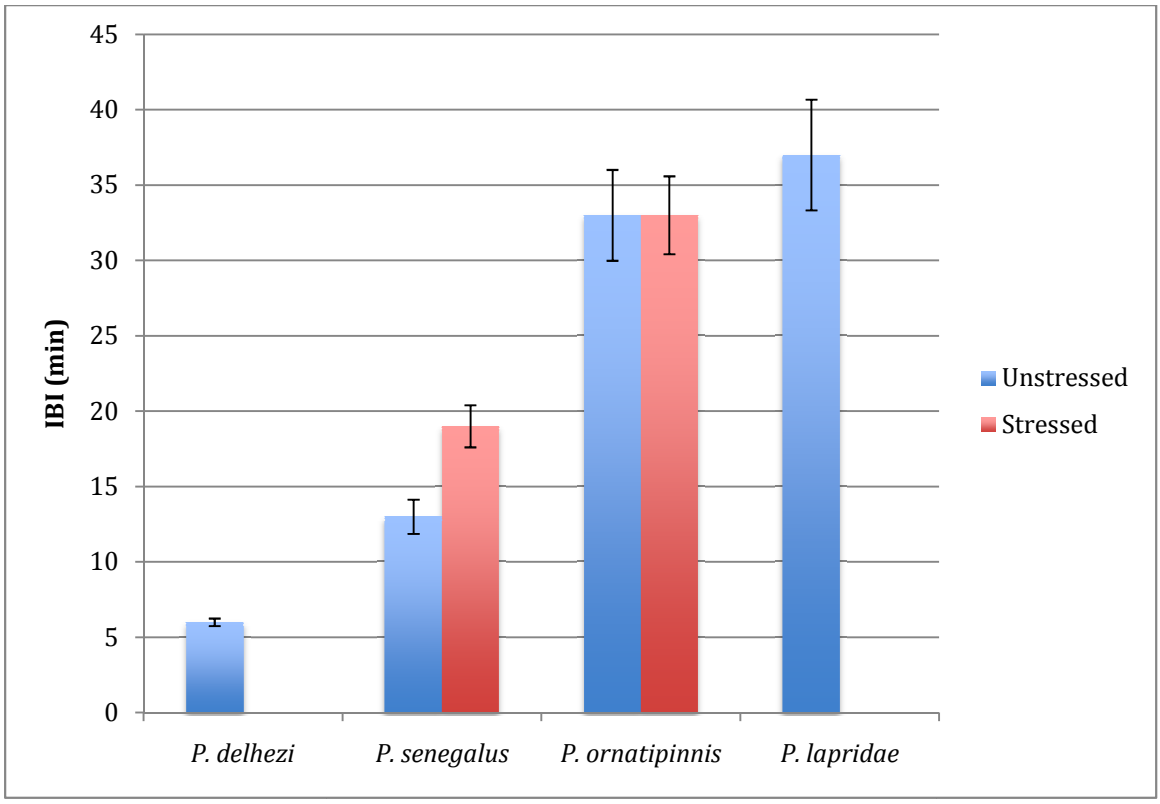

Figure 6: Average ($\bar{x}$ ± SE) IBI for each fish under each condition, stressed and unstressed.

Table 11: Average ($\bar{x}$ ± SE) IBI value for each of the six fish and both experimental conditions.

	Average IBI (min)
P. delhezi, unstressed	6 ± 0.2503
P. senegalus, stressed	19 ± 1.3912
P. senegalus, unstressed	13 ± 1.1407
P. ornatipinnis, stressed	33 ± 2.5894
P. ornatipinnis, unstressed	33 ± 3.0146
P. lapradei, unstressed	37 ± 3.6697

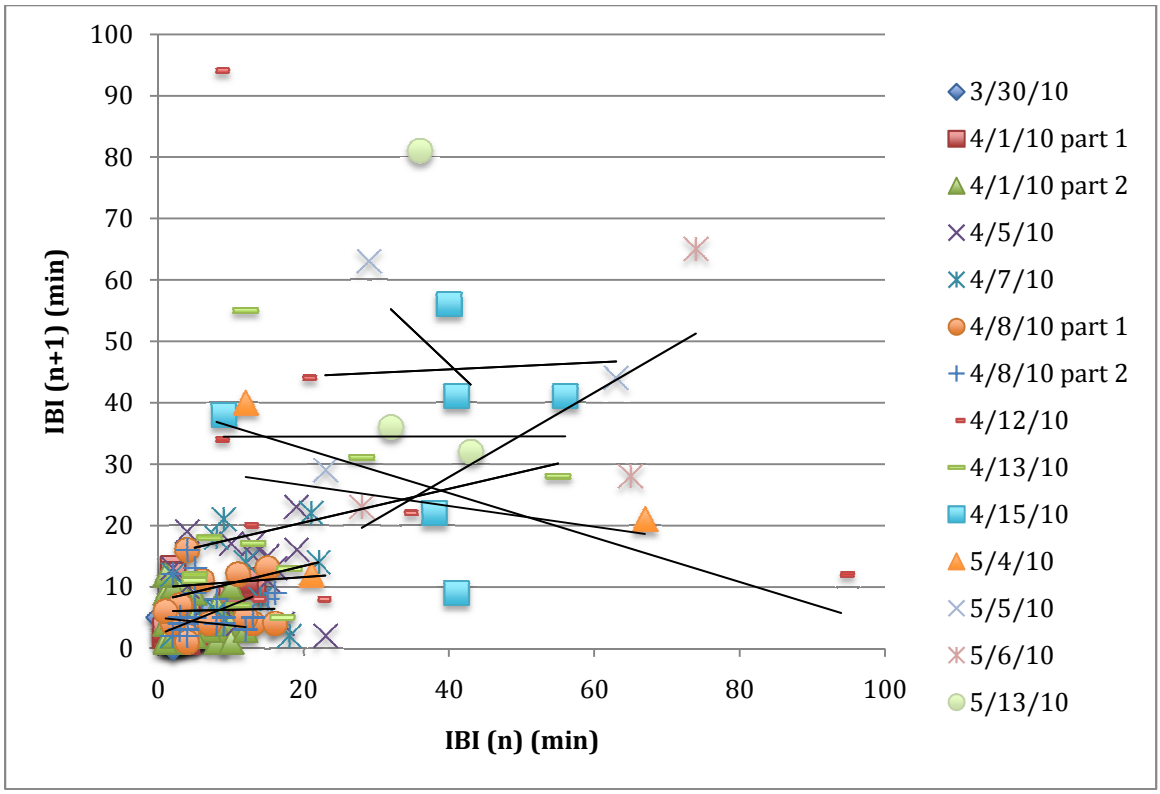

Figure 7: Poincaré plots of IBIs for *P. senegalus*, unstressed for individual observation periods. The individual observation periods have the following regression line and R-squared value: 3/30/10 ($y = -0.1073x + 4.4221$, $R^2 = 0.011$), 4/1/10 part 1 ($y = 0.4747x + 2.2661$, $R^2 = 0.1951$), 4/1/10 part 2 ($y = -0.127x + 5.0129$, $R^2 = 0.0164$), 4/5/10 ($y = 0.0849x + 9.9026$, $R^2 = 0.0075$), 4/7/10 ($y = 0.2839x + 7.7826$, $R^2 = 0.0755$), 4/8/10 part 1 ($y = 0.1485x + 5.4898$, $R^2 = 0.027$), 4/8/10 part 2 ($y = 0.0283x + 6.0103$, $R^2 = 0.001$), 4/12/10 ($y = -0.3621x + 39.8$, $R^2 = 0.1309$), 4/13/10 ($y = 0.2744x + 15.008$, $R^2 = 0.0761$), 4/15/10 ($y = 0.0013x + 34.453$, $R^2 = 1E{-}06$), 5/4/10 ($y = -0.1691x + 29.97$, $R^2 = 0.1218$), 5/5/10 ($y = -0.1691x + 29.97$, $R^2 = 0.1218$), 5/6/10 ($y = 0.687x + 0.4212$, $R^2 = 0.533$), and 5/13/10 ($y = -1.1129x + 90.844$, $R^2 = 0.0519$).

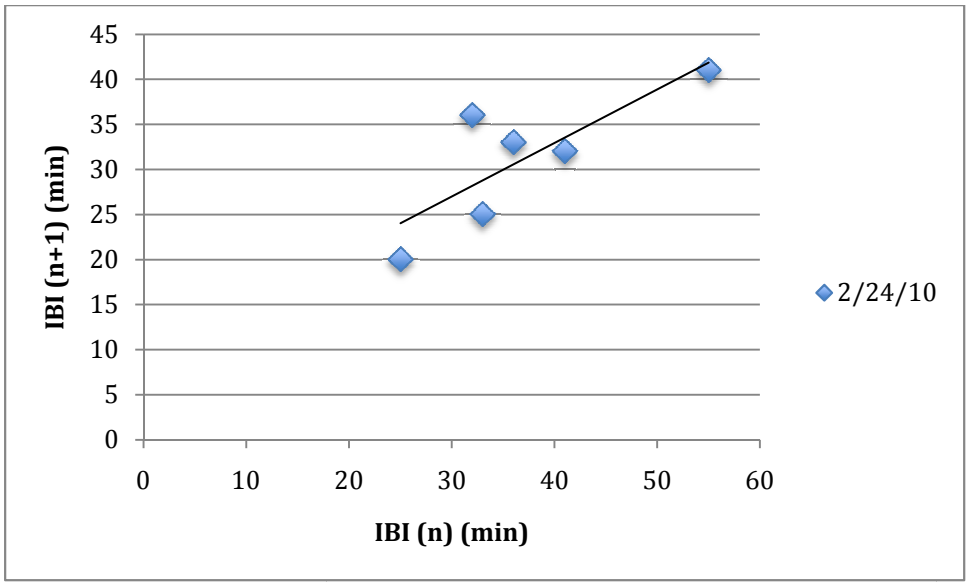

Figure 8: Poincaré plot of IBIs for *P. ornatipinnis*, unstressed for individual observation periods. The individual observation period has the following regression line and R-squared value: 2/24/10 ($y = 0.5851x + 9.1496$, $R^2 = 0.64934$.)

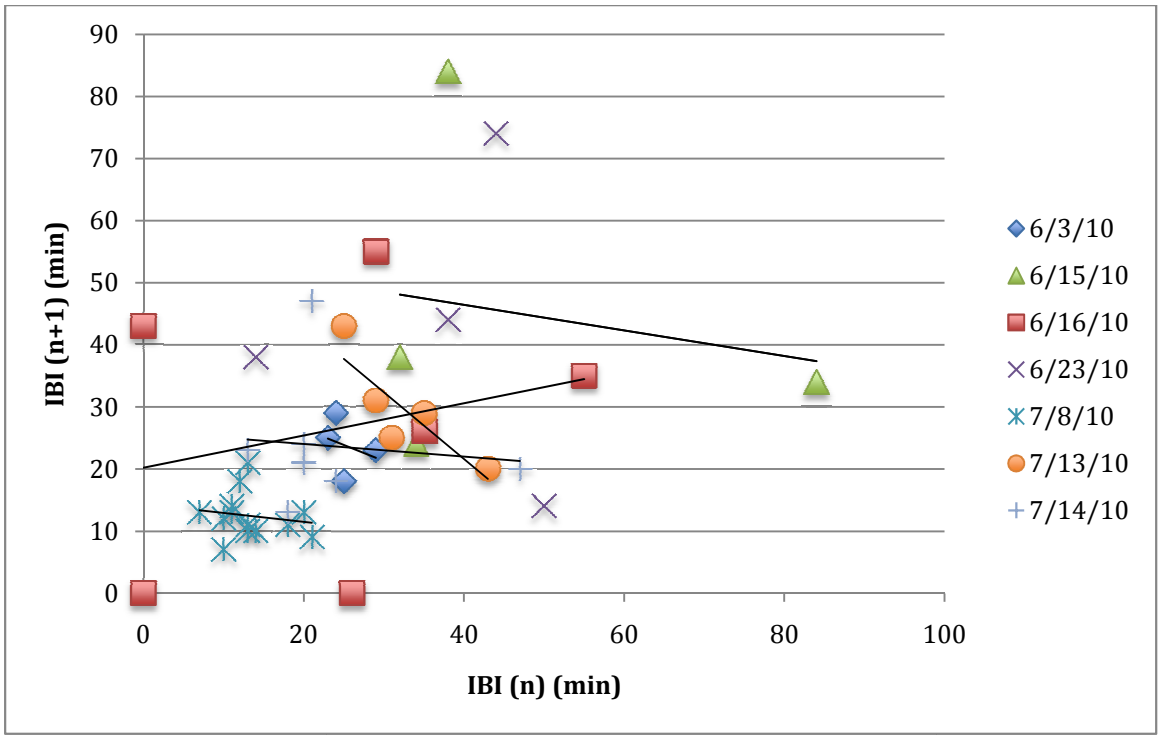

Figure 9: **Poincaré plot of IBIs for *P. lapradei*, unstressed for individual observation periods.** The individual observation periods have the following regression line and R-squared value: 6/3/10 ($y = -0.5181x + 36.831$, $R^2 = 0.08875$), 6/15/10 ($y = -0.2061x + 54.685$, $R^2 = 0.03673$), 6/16/10 ($y = 0.2596x + 20.226$, $R^2 = 0.05955$), 6/23/10 ($y = -0.0602x + 44.699$, $R^2 = 0.00148$), 7/8/10 ($y = -0.1437x + 14.374$, $R^2 = 0.02508$), 7/13/10 ($y = -1.0726x + 64.568$, $R^2 = 0.72963$), and 7/14/10 ($y = -0.1015x + 26.078$, $R^2 = 0.01048$).

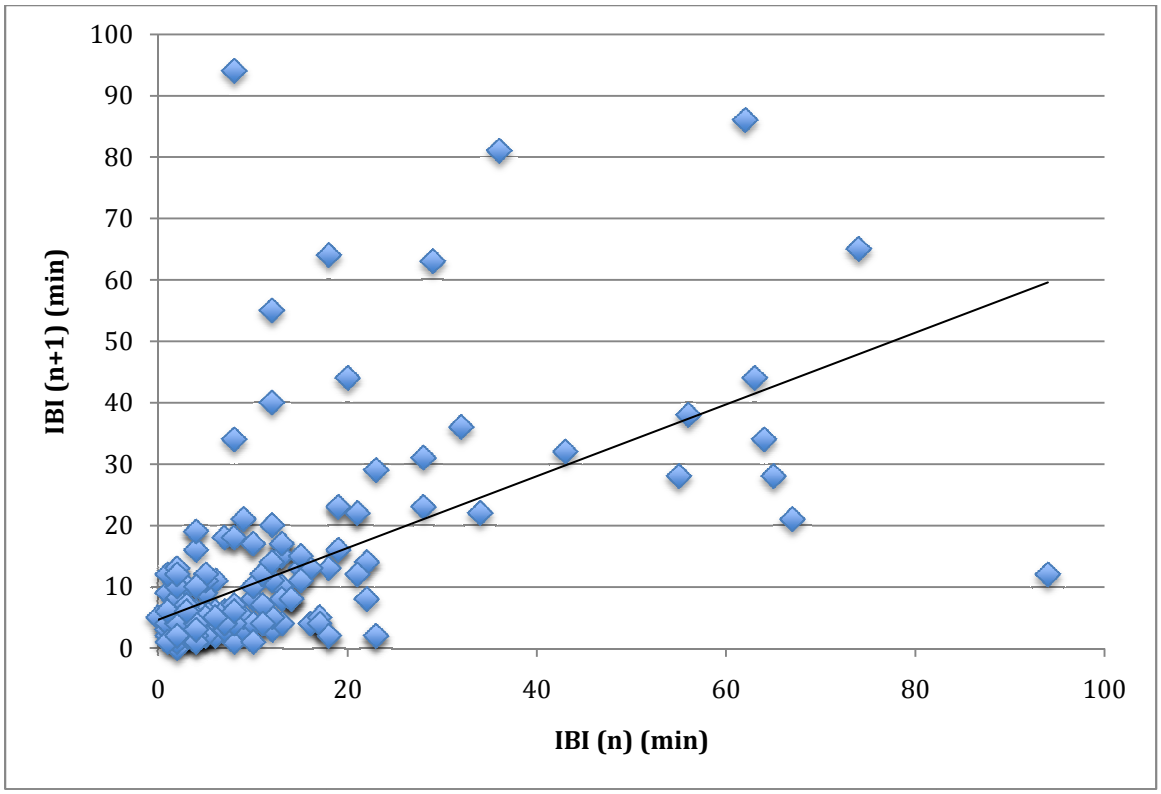

Figure 10: Poincaré plot of IBIs for *P. senegalus*, unstressed for all observation periods combined. The regression line and R-squared value for these data are: y = 0.5843x + 4.7008, R^2 = 0.3234.

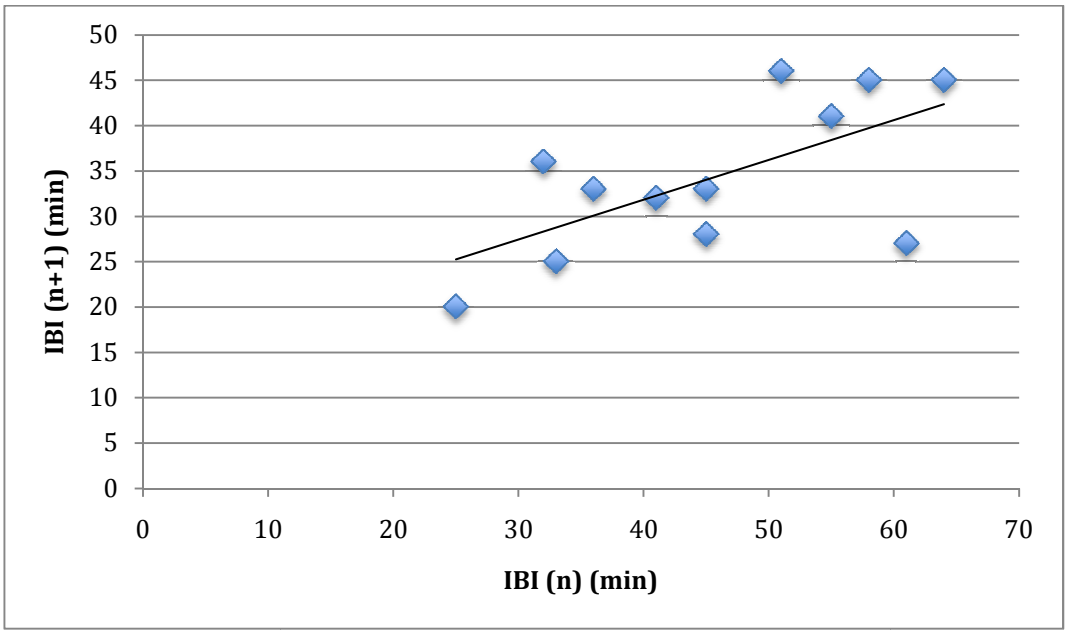

Figure 11: Poincaré plot of IBIs for *P. ornatipinnis*, unstressed for all observation periods combined. The regression line and R-squared value for these data are: y = 0.4393x + 14.263, R^2 = 0.4138.

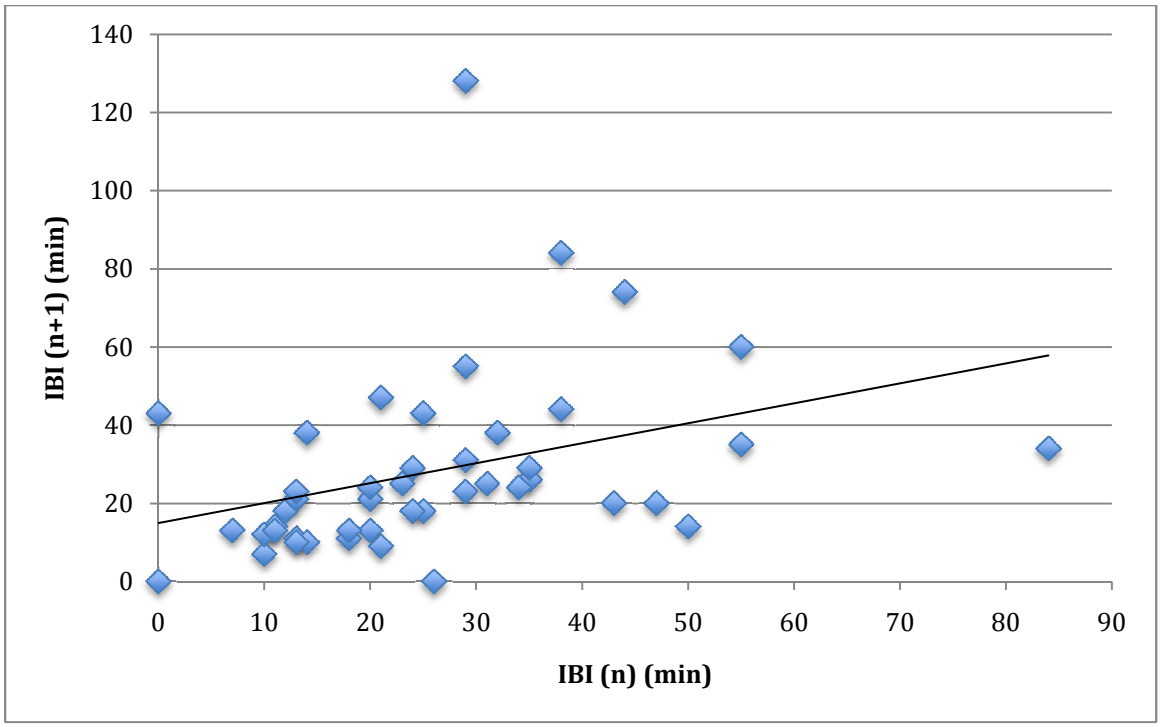

Figure 12: Poincaré plot of IBIs for *P. lapradei,* unstressed for all observation periods combined. The regression line and R-squared value for these data are: y = 0.5113x + 14.963, R^2 = 0.12374.

Breath Cycle

Examination of videos of *P. delhezi* (140.70 g, total length = 29.7 cm) and *P. senegalus* (34.75 g, total length = 19.8 cm) breaths, identified the timing of key events in the air breath cycle of *Polypterus*. Key events are defined as actions that consistently occur, always in the same order, during every air breath. These include: 1) the head being parallel to the water surface, 2) the operculum expanding, 3) the floor of the mouth dropping, 4) the opening of the spiracular valve, 5) the floor of the mouth dropping a second time with the release of the previous air breath, and 6) the closing of the spiracular valve. Both species examined had the same order of key events in their breath cycle.

Schematics of these events were constructed for both *P. delhezi* and *P. senegalus* (Figures 13 and 14) using the average time of each event's occurrence with the first event set at 0.00 s. The averages for *P. delhezi* were based on ten individual breaths and are as follows: 1) the head being parallel to the water surface = 0.00 s, 2) the operculum expanding = 0.28 ± 0.08 s, 3) the floor of the mouth dropping = 0.34 ± 0.09 s, 4) the opening of the spiracular valve = 0.43 ± 0.09 s, 5) the floor of the mouth dropping a second time = 0.52 ± 0.10 s, and 6) the lowering of the spiracular valve = 0.54 ± 0.09 s. The averages for *P. senegalus* were based on four individual breaths and are as follows: 1) the head being parallel to the water surface = 0.00 s, 2) the operculum expanding = 0.03 ± 0.01 s, 3) the floor of the mouth dropping = 0.04± 0.01 s, 4) the opening of the spiracular valve = 0.15± 0.01 s, 5) the floor of the mouth dropping a second time = 0.18 ± 0.01 s, and 6) the lowering of the spiracular valve = 0.24 ± 0.02 s. In general, the breath cycle of *P. delhezi* was longer than that for *P. senegalus*, though both showed a high level of variation in the timing of each event.

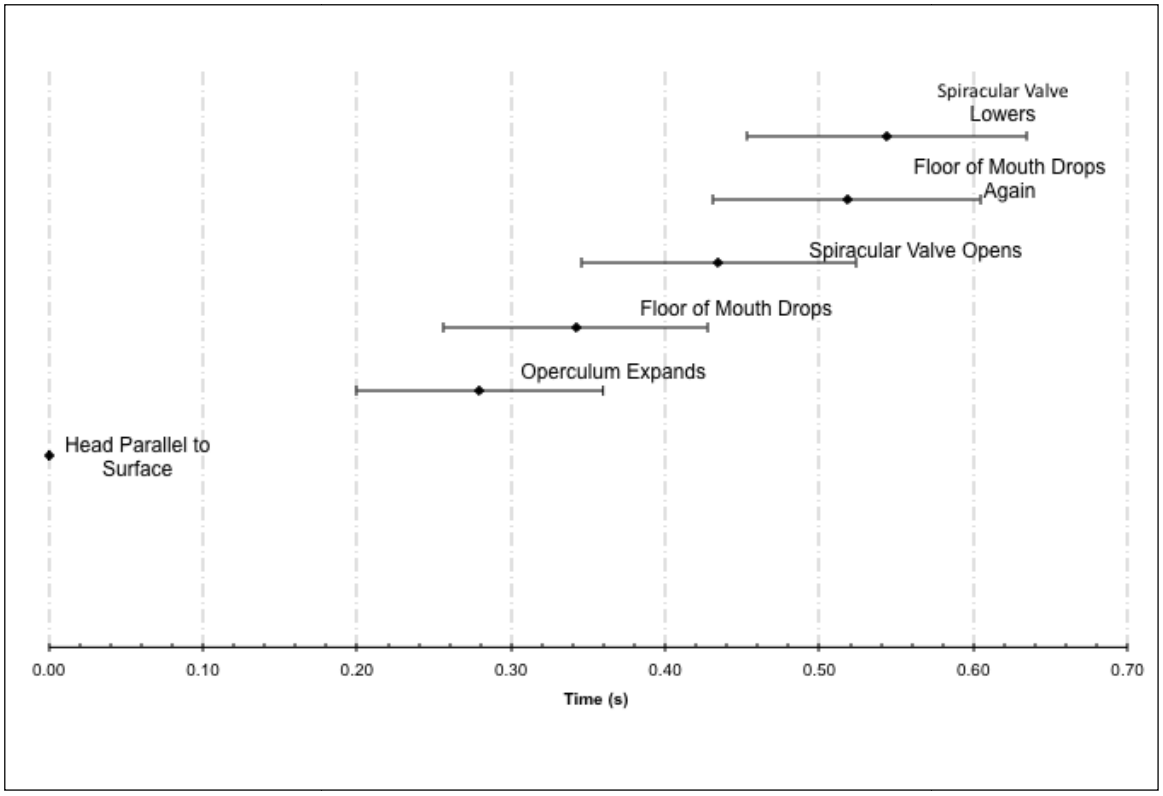

Figure 13: *P. delhezi* activity series showing the average ($\bar{x}$ ± SE) timing of the key events of the breath cycle. Average based on ten recorded breaths, reported as both a date and time of breath: 3/30/09 (1315), 3/30/09 (1341), 4/1/09 (1353), 4/1/09 (1419), 4/2/09 (1500), 4/2/09 (1553), 4/6/09 (1357), 4/7/09 (1518), and 4/7/09 (1559).

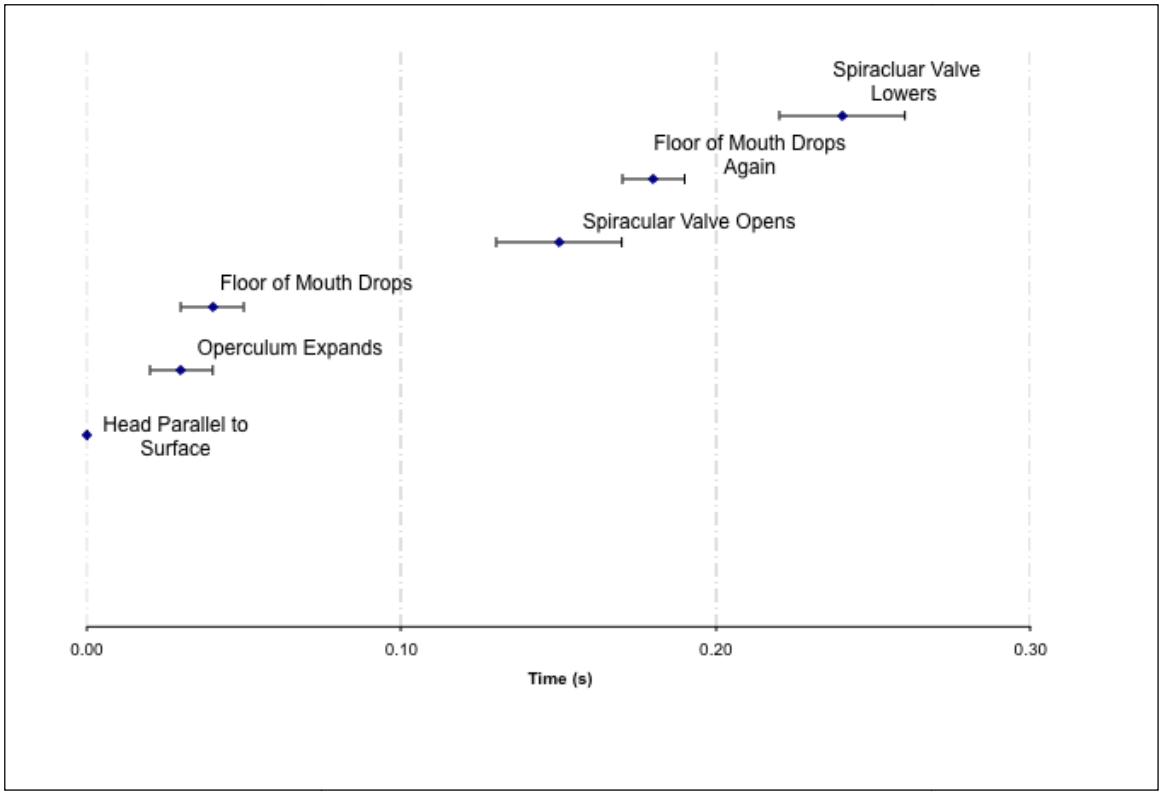

Figure 14: ***P. senegalus*** **activity series showing the average ($\bar{x}$ ± SE) timing of the key events of the breath cycle.** Average based on four recorded breaths, reported as both a date and time of breath: 4/22/09 (1238), 5/12/09 (1530), 5/13/09 (1412), and 10/21/09 (1327).

The average timing between key events, including the time between the floor of the mouth dropping for the first time and the spiracular valve first being visible and the time between the operculum expanding and the spiracular valve first being visible were also calculated with a standard error to consider whether the timing of the breath cycle was conserved between different *Polypterus* species (Figure 15). The average time between the floor of the mouth dropping the first time and the spiracular valve first being visible above the waterline was 0.09 ± 0.06 s for *P. delhezi* and 0.11 ± 0.02 s for *P. senegalus,* and the average time between the operculum expanding and the spiracular valve first being seen was 0.16 ± 0.06 s for *P. delhezi* and 0.12 ± 0.03 s for *P. senegalus*.

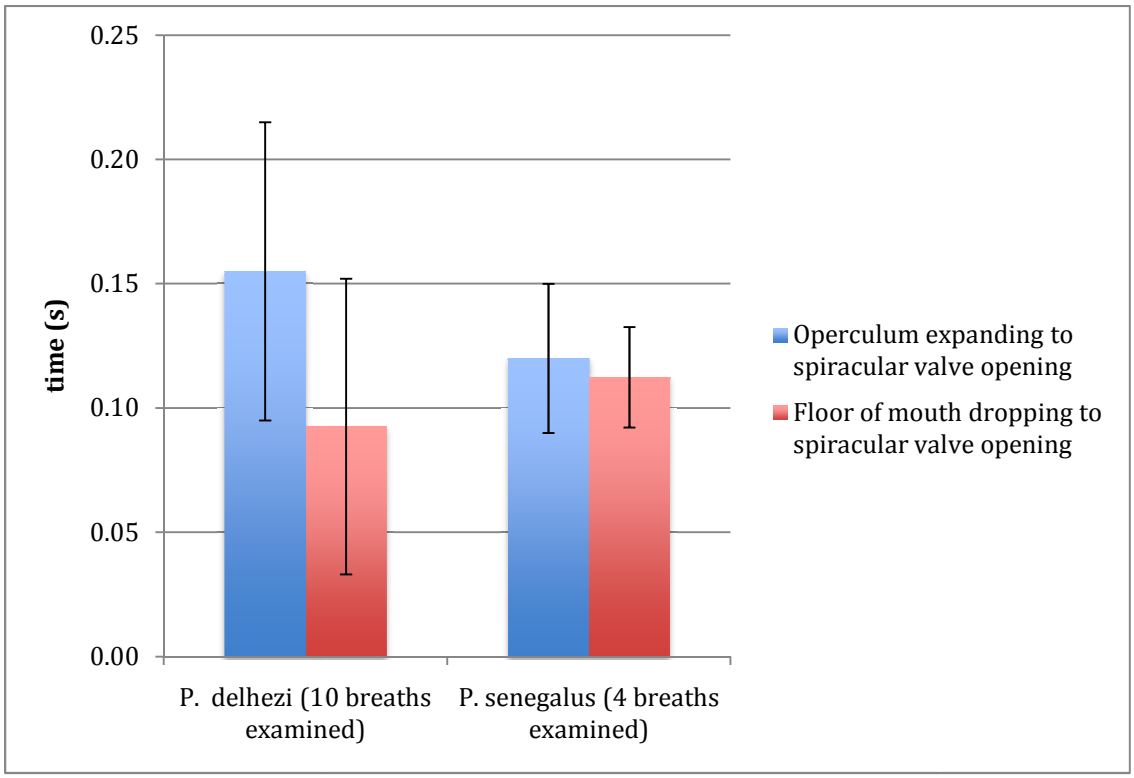

Figure 15: Average (± SE) timing between key events of the breath cycle. This includes the time between the floor of the mouth dropping and the first sighting of the spiracular valve and the time of the operculum expanding and the first sighting of the spiracular valve both for *P. delhezi* (Ten breaths reported as both a date and time of breath: (3/30/09 (1315), 3/30/09 (1341), 4/1/09 (1353), 4/1/09 (1419), 4/2/09 (1500), 4/2/09 (1553), 4/6/09 (1357), 4/7/09 (1518), and 4/7/09 (1559)) and *P. senegalus* (Four breaths: 4/22/09 (1238), 5/12/09 (1530), 5/13/09 (1412), 10/21/09 (1327)).

Another event that always occurs during the breath cycle is the exhalation of the previous breath. This results in the release of an air bubble via the operculum. For *P. delhezi* the average timing of bubble release is 0.33 ± 0.09 s after surfacing and for *P. senegalus* it occurs at 0.07 ± 0.05 s. The location of this event within the breath cycle differs between the two species examined. *P. delhezi* usually (70% of the breaths observed) has the release of the bubble directly following operculum expansion (step two), but in 30% of the breaths observed bubble release occurred after dropping of the

floor of the mouth (step three). For *P. senegalus*, the air bubble was always released following the floor of the mouth dropping the first time (step three).

Dissections

Dissections allowed a close examination of spiracle structure. Details of the final two fish examined are as follows. Both samples had two pre-spiracular ossicles, two spiracular ossicles, and three post-spiracular ossicles. Also found was a small muscle that lies directly underneath and is attached to the spiracular ossicles (Figure 16). This muscle had not been documented in previous research of *Polypterus* anatomy. The *dilatator operculi* was identified in both specimens, but the *musculus spiracularis* could not be definitively identified in either fish (Figure 17). A section of the *dilatator operculi* that could have possibly been the *musculus spiracularis* was seen in one of the fish, but not the other. Additionally, there was no visible connection between any part of the *dilatator operculi* and the spiracular ossicles, and no obvious method by which it might control the opening or closing of the spiracle.

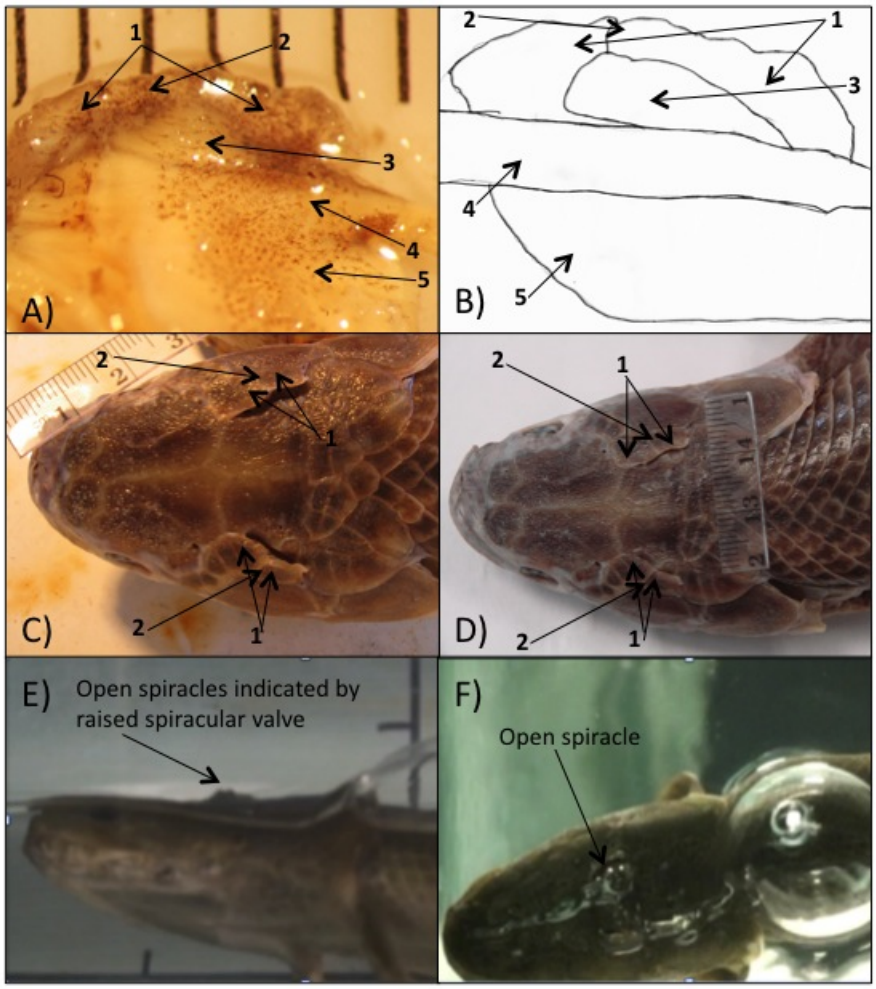

Figure 16: Overview of the muscles and bones involved in spiracular opening and closing. The numbers correspond to the following: 1) Spiracular ossicles, 2) Spiracular joint, 3) Small, unknown muscle found directly below the spiracular ossicles, 4) *Dilatator operculi*, and 5) *Musculus protractor hyomandibularis*. A) Microscope photo of the unknown muscle and its position relative to the spiracular ossicles. B) Sketch representation of the unknown muscle. C) *P. ornatipinnis* with open spiracles. A piece of paper was inserted below the ossicles to represent the contracted muscle and how it could open the spiracles at the spiracular joint. D) *P. ornatipinnis* with closed spiracles. E) Side view of an air-breath with a raised spiracular valve. F) Top view of an air-breath with open spiracles.

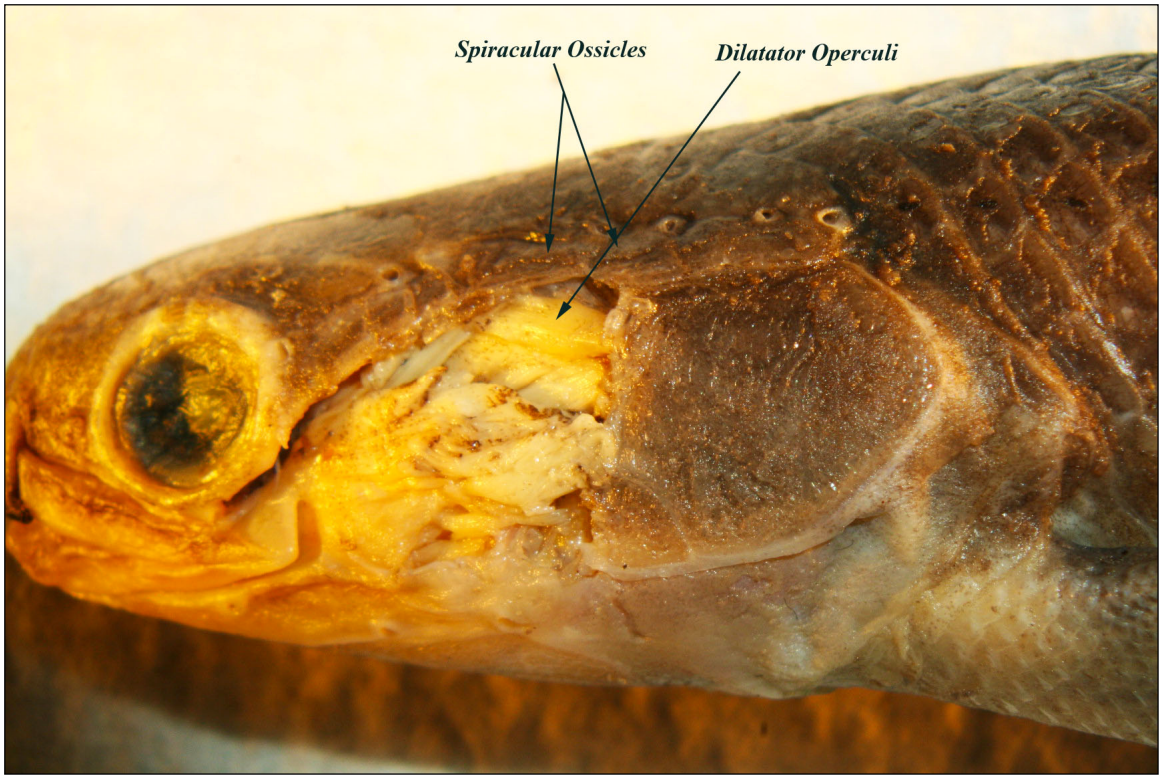

Figure 17: *Polypterus* cranial anatomy, including the *dilatator operculi* and the spiracular ossicles.

Discussion

Spiracle Use

Stressed vs. Unstressed Conditions

This study demonstrates that *Polypterus* can use its spiracles for aerial inspiration. Videos of all four species during air breathing confirm the use of spiracles to breathe air when the mouth remains closed and submerged. These results thus confirm the early reports of Budgett (1903) and Magid (1966), but are in direct conflict with those by Brainerd et al (1989), which stated that *Polypterus* (specifically *P. senegalus)* does not use its spiracles for inhalation. Air-breathing via the mouth is most often associated with stressed and "unnatural" conditions common in laboratory experiments in which precautions are not taken to permit the fish to approach the water surface with minimal disturbance. Also, the percentage of spiracular air breaths was higher in unstressed conditions than in stressed conditions. This suggests that the natural air-breathing behavior of *Polypterus* is to inhale via the spiracle.

This provides a possible explanation for Brainerd et al.'s (1989) conclusion that the spiracle was not used for air inhalation. If the spiracle is most commonly used in unstressed or "natural" conditions and inhalation via the mouth is done when the fish is in a stressful or unnatural situation, it is reasonable to assume that the conditions the fish were in for their reported experiments were more similar to stressed than unstressed conditions and thus no air-breathing via spiracle was observed. The goal of their experiment was not to examine the role of spiracles, thus they never optimized the conditions for spiracle breathing and thus did not see the high levels of spiracle use observed in this study.

Additionally, some of the experiments reported by Brainerd et al. (1989) may have been performed at an incline and this could have influenced spiracle use. In order for *Polypterus* to utilize its spiracle to breathe air, its head must be parallel to the surface of the water. In contrast, during mouth air breaths the fish approaches the surface and breathes at an angle. If the fish is at an incline, it effectively prevents the fish from approaching the surface horizontally and breathing from its spiracle. Instead, it would use its mouth to breath, as these researchers observed. This angled approach during air-breathing with the mouth may be a less efficient breathing method due to the increased hydrostatic pressure the fish must contend with to fill its lung. From x-ray video frames in Brainerd and Ferry-Graham (2005), one can extrapolate that the angle the fish makes with the water is on average approximately 66°. With this angle of approach, a *Polypterus* of length 20.0 cm with a 15.0 cm lung, the fish would need to overcome an additional 0.01326 atm of pressure to reach the bottom of the lung and this would effect it's ability to fill the lung completely. A fish that is parallel to the water surface would not have to overcome the same hydrostatic pressure in order to fill the lung completely. Figure 1-10 in Brainerd and Ferry-Graham (2005) further emphasizes the possible inefficiency of mouth breaths. Radiographs show only partial filling of the lung during an air breath.

Levels of Variation

Even though the unstressed and stressed conditions markedly influenced spiracle use, there was still a relatively large variation in the percentage of spiracle breaths per observation period within the same fish and experimental condition. With the same fish, experimental conditions, and similar temperature and oxygen levels, the observation

period breakdowns reveal that the percent spiracle use can still be different (Table 5-10). One source of this variation could be disturbances that were impossible to eliminate, such as ambient noise and vibrations. These would differ from one observation period to the next, and could not be standardized, and may thus have contributed to variations in spiracle use. This further supports the idea that behavior-affecting circumstances in the natural environment and the laboratory strongly influence the degree to which *Polypterus* utilizes its spiracles compared to its mouth for inhalation.

P. delhezi

Because the *P. delhezi* individual observed in this research is blind it acts independently of visual disturbances and teaches one a great deal about the effect of disturbances on *Polypterus* breathing behavior. It showed the "natural" use of spiracles for air-breathing even before the development of an optimal tank setup. This first fish observed, and its use of spiracles for aerial respiration became the basis of this study. Without this fish, the initial observations of spiracle use may not have been seen and the idea of environmental disturbances created in the laboratory environment affecting breathing behavior would not have been developed. If one of the other fish had been observed first without knowing the impact disturbance levels have on spiracle use, it would have led one to believe that the spiracles were used rarely, while in actuality they are preferentially used in natural conditions.

Mouth Air Breaths

A possible explanation of the predominance of mouth air breaths during stressed conditions could be the faster speed of these breaths when compared to spiracle air breaths. If a fish feels threatened the goal would be to take as quick of a breath at the

surface as possible, and then return to deeper water. Observations show that the fish spends less time at the water surface during a mouth air breath than a spiracle air breath, thus making aerial inspiration via the mouth faster and the preferred method of inhalation in stressed conditions.

Breathing Frequency

Environmental Effects

The most notable observation of the air-breathing frequency data is ta frequency independence from all of the environmental factors manipulated in this study, suggesting instead that it is a characteristic of each individual fish. Temperature and oxygen were expected to influence the air-breathing rate of *Polypterus*, but had little effect. One would expect that the further into an observation period the fish was, the more frequent air-breathing would be due to the lowered oxygen level and the increased temperature, but this was not seen. Additionally, the frequency of air breaths does not appear to be reliant on disturbance level. Unlike the use of spiracles for aerial respiration, there was no overall pattern seen when considering breathing rate differences between stressed and unstressed conditions (Figure 5, Table 4).

IBIs

Poincaré plots of IBIs also proved ineffective at describing the air-breathing frequency of *Polypterus* for individual observation periods. One would expect that the time between a single air breath and the breath before it would be related to the time between the same single breath and the breath right after it, but all but one (unstressed *P. ornatipinnis*, 2/24/10) of the lines of identity for individual observation periods showed no correlation (Figure 7–9). The 2/24/10 observation period has a relatively strong

correlation between IBIs, with a clear line of identity. The reasons why the 2/24/10 observation period for *P. ornatipinnis* showed a relationship between IBIs, while the others did not are unknown, though it could be related to the variability associated with IBIs within one observation period (Figure 7–9). Poincaré plots for lumped sets of IBIs showed clear lines of identity with relatively high R-squared values for both *P. senegalus* and *P. ornatipinnis*, but not for *P. lapradei* (Figures 10–12). Again, it is unclear why a relationship between IBIs would be present in some species of *Polypterus*, but not in others. There seems to be some relationship between the timing between air breaths at least on a species level, but it is highly variable and may not be seen for all *Polypterus*. The fact that lumped IBIs show a relationship, while IBIs from a single observation period usually do not, indicates that IBIs and air breathing frequency are a characteristic of a given fish and are independent of environmental factors that vary on an observational period level.

IBIs also show varying relationships to stressed and unstressed conditions. Both *P. senegalus* and *P. ornatipinnis* were observed in both of the conditions. *P. ornatipinnis* has almost the exact same average IBI value for both stressed and unstressed conditions (stressed: 32 ± 0.0018 min, unstressed: 32 ± 0.0021 min). In contrast, *P. senegalus* has a higher average IBI value, 18 ± 0.0010 min vs. 12 ± 0.0008 min, in unstressed vs. stressed conditions (Table 11). The different correlations of IBIs to environmental conditions in fish of two different species emphasize the likelihood that air-breathing frequency is a unique characteristic for each *Polypterus* species.

Species Comparison

Instead of being dependent upon environmental factors, the average air-breathing frequency and IBI value for *Polypterus* may be a characteristic of each species. The data reveal that each species observed in this study had a different breathing frequency pattern associated with it (Figure 5, Table 4). There is a vast range of average breathing rates, with *P. delhezi*, unstressed having the highest average breathing frequency associated with it (12.48 ± 1.478 breaths per h per observation period) and *P. ornatipinnis*, unstressed having the lowest (0.63 ± 0.16 breaths per h per observation period). IBIs showed a similar pattern, with each species having its own IBI value (Figure 6, Table 11). IBI values ranged from 6 ± 0.0002 min (*P. delhezi*) to 37 ± 0.0029 min (*P. lapradei*).

Additionally, a different general pattern of air-breathing rate was found for the two species observed in both experimental conditions. While *P. ornatipinnis* has a lower breathing rate in unstressed than in stressed conditions, *P. senegalus* has a higher rate in unstressed than in stressed conditions (Figure 5, Table 4). This could be due to the fact that environmental factors affect some of the species, but not others. This appears to hold true for average IBI values as well, with one species affected by environmental conditions (*P. senegalus*), but not the other (*P. ornatipinnis)* (Figure 6, Table 11).

Levels of Variation

Much like for spiracle use, the air-breathing rate had some variability even when considering the same fish and condition (Table 4). The source of this variability is hard to pinpoint, and it is likely not due to uncontrollable disturbances, since disturbance level (stressed vs. unstressed) doesn't seem to have a consistent effect on air-breathing frequency. The variation may again be a characteristic of the specific fish used in this

study. It is also important to note that compared to both spiracle use and air-breathing rate, average IBIs showed relatively low variability, as indicated by their small coefficients of determination (Table 11).

Future Research

This research was unable to find or explain any patterns of air-breathing frequency. Further examination of breathing frequency, perhaps with replicates of each species should be performed in order to gain more insight into air-breathing rates and whether they are a species- or individually-specific. Additionally, air-breathing frequency should be assessed in conjunction with the volume of oxygen inhaled with each breath. Preliminary research quantifying the amount of oxygen inhaled with each breath using an Oxzilla oxygen meter, show that the amount of oxygen inhaled differs between breaths. This leads one to hypothesize that the number of breaths might be correlated with the amount of oxygen inhaled. This could account for the amount of variation seen in air-breathing frequency and could explain why air-breathing rate did not increase with increased water temperature or decreased levels of dissolved oxygen.

Breath Cycle

Determination of the Breath Cycle

A distinct breath cycle was seen for all examined spiracle air breaths, with six events that always occurred, and it was conserved in both species examined (*P. delhezi* and *P. senegalus*). This further supports the idea that spiracle air-breathing is the more "natural" breathing method, as stereotypical patterns and cycles are commonly seen in natural animal behavior.

Timing of the Breath Cycle and Variation

All six steps identified as part of the breath cycle show a relatively high level of variation in the time that they occurred for both of the species examined (Figure 13 and Figure 14). Additionally, the total length of the cycle varies from one breath to another. This indicates that although there is a clear series of events, the breath cycle is still somewhat irregular even when considering an individual fish. Release of the air bubble at the start of the breath also shows variable of timing and location within the breath cycle, sometimes occurring right after the operculum expands and other times occurring after the first time the floor of the mouth drops. These events usually occur within tenths of seconds of each other, so bubble release is still limited to a small time range within the cycle.

When looking specifically at the time between the operculum expanding and the first visibility of the spiracular valve and the floor of the mouth dropping and the first visibility of the spiracular valve, the two species observed show differing levels of variability. *P. senegalus* shows less variation than *P. delhezi*, however this is likely only an artifact of the smaller sample size (four breaths vs. ten breaths) (Figure 15). Continued observation and examination of more breaths could be performed to help resolve this issue, as well as observations of additional fish of the two species examined. Differences between species suggest that although the breath cycle is conserved from one species of *Polypterus* to another, the timing is not.

Bubble Release

Bubble release was not included as a definite "event" of the breath cycle due to its varying appearance within the breath cycle of the two different species, and even among different *P. delhezi* breaths. In the four breaths analyzed for *P. senegalus,* bubble release from the spiracle always occurred directly following the floor of the mouth dropping the first time (step two of the breath cycle). However while in 30% of observed breaths *P. delhezi* bubble release occurred in the same position of the breath cycle as in *P. delhezi*, bubble release in *P. delhezi* occurred more frequently between the expansion of the operculum and the dropping of the floor of the mouth (70% of observed breaths).

Dissections

Muscle Observations

Dissection of *Polypterus* cranial musculature gave new insight into the mechanism by which the spiracular valve might function. Allis (1922) describes the *musculus spiracularis* as having attachments to the spiracular ossicles of the spiracular valve, but these observations saw no direct connections to the spiracular ossicles and gave no indication that it could perform the function of opening and closing the spiracular valve. Attempts to manipulate the *dilatator operculi* did not appear to open or close the valve (Figure 17). The literature disagrees on whether the *musculus spiracularis* as a separate muscle running parallel to the *dilatator operculi* or a "slip" of the *dilatator operculi,* and in dissection performed in this study it was never observed as a separate muscle. This led to the search for a more likely candidate.

A small muscle was found underneath the spiracular ossicles and it could be responsible for opening and closing the spiracular valve. The existence of this muscle had

not been previously recorded, but it has the potential to be important in the study of spiracle function. Unlike the *musculus spiracularis,* this muscle has direct attachments to both of the spiracular ossicles making it a more likely candidate for controlling the spiracle than the *musculus spiracularis* or *dilatator operculi* (Figure 16 A–B).

Possible Mechanism of Spiracular Valve Opening/Closing

The structure and location of the newly discovered muscle allows a possible mechanism for spiracular valve opening and closing to be generated. When this muscle is relaxed the two spiracular ossicles lie flat and form a seal over the spiracle. In this state, the spiracle is closed (Figure 16 D). However, when the muscle contracts, the plates are pulled together at the spiracular joint, and the spiracular valve opens (Figure 16 C). When raised the joint between the two spiracular ossicles is bent indicating the shortening of the muscle (Figure 16 E–F). The muscle is not visible during an air-breath but the raised spiracular valve is evident in a side-view of the fish (Figure 16 E) and the open spiracles from a top-view of the fish (Figure 16 F).

Muscles Involved in the Breath Cycle

The events in the breath cycle allow one to determine the muscles that play a role in air-breathing beyond those that specifically open the spiracle. The role of the small, unknown muscle has already been described and it may be responsible for the lifting of the spiracular ossicles and the opening of the spiracle. It would contract during step four of the breath cycle, opening the spiracle and raising the spiracular valve, and it would relax in step six of the breath cycle when the spiracle closes and the spiracular valve lowers. Additionally, the opercula expand and the floor of the mouth drops twice. Opercula expansion is performed by *levitator opercula* muscles, including the *dilatator*

operculi (visible in Figure 16 A–B). These muscles would be playing an active role in the breath cycle during the second step. The lowering of the mouth, an essential part of the breath cycle, involves abductor mandibular muscles, including the *musculus protractor hyomandibularis* (Figure 16 A–B), and the geniohyoideus muscle. Hypaxial muscles are also involved in the depression of the mandible (Helfman et al., 2007). These muscles would thus be directly involved in steps three and five of the breath cycle when the floor of the mouth is lowered. The small muscle discovered in dissections performed appears to be closely associated with many of these other muscles that would play a role in air-breathing (Figure 16 A–B)

Conclusion

This research shows that *Polypterus* utilizes its spiracles to inhale air. Over 580 individual spiracle air breaths were observed for four different species of *Polypterus*, most of which were under unstressed conditions. This study reveals that the use of the spiracle for air-breathing is highly dependent on the disturbance levels, and the spiracle is used preferentially when disturbance levels are low (i.e. unstressed conditions), but rarely when disturbance levels are high. A distinct breath cycle for spiracle air breaths was also found. It is conserved between two P*olypterus* species and has six key events that always occur. Additionally, studies show that neither temperature or oxygen n\or disturbance levels appear to play a direct role in influencing air-breathing rate, despite expectations that they would. Poincaré plots show an overall correlation between one IBI and the subsequent IBI for 2/3 of the fish species examined (specifically *P. senegalus* and *P. ornatipinnis*), but very few relationships between breaths in individual observation periods, further compounding the mystery of what affects the breathing frequency of *Polypterus*. Future research, perhaps with a quantitative measurement of inhalation volume, may give more insight into what effects how frequently these fish breathe. Dissections performed as part of this research indicated that the *musculus spiracularis* is not involved in the opening and closing of the spiracular valve. Instead, a newly observed muscle extending along the ventral surface of the spiracular ossicles may perform this role. This muscle had not been previously reported, and a possible mechanism of how it opens and closes the spiracular valve was derived. This research provides not only proof of spiracular use in *Polypterus* for respiration, but also a unique starting point for future

research on its air-breathing physiology as well as general spiracle structure. Additionally, spiracles are important structures that were also present in late Devonian lobefin fishes and early tetrapods that breathed air (Graham, 2006). Thus, the use of spiracles in *Polypterus* for air breathing may provide new insight into how early tetrapods may have used their spiracles and a similar “recoil aspiration” breathing method to breath air.

Literature Cited

Allis Jr., E. P. (1922). The cranial anatomy of *Polypterus* with special reference to *Polypterus bichir*. *J. Anat. Lond.* 56, 189–224.

Barry, M. A, D. H. Hall, & Bennett, M. V. L. (1987). The elasmobranch spiracular organ. *J. of Comp. Physiol.* 163, 85–92.

Brainerd, E. L., Liem, K. F., & Samper, C. T. (1989). Air ventilation by recoil aspiration in polypterid fishes. *Science* 246, 1593–1595.

Brainerd, E. L., & Ferry-Graham, L. A. (2005). Mechanics of respiratory pumps. *Fish Physiol.* 23, 1–28.

Britz, R. & Johnson, G. D. (2003). On the homology of the posteriormost gill arch in polypterids (Cladistia, Actinopterygii). *Zool. J. Linn. Soc.* 138, 495–504.

Budgett, J.S. (1903). Notes on spiracles of *Polypterus. Proc. Zool. Soc. Lond.*, 10–11.

Burggren, W., Johansen, K., & McMahon, B. (1985). Respiration in phyletically ancient fishes. In *Evolutionary Biology of Primitive Fishes.* Eds. Foreman, R.A., Gorbman, A., Dodd, J.M., Olsson, R. New York: Plenum Press, 217–252.

Claeson K.M., Bemis, W.E., & Hagadorn, J.W. (2007) New interpretations of the skull of a primitive bony fish *Erpetoichthys calabaricus* (Actinopterygii: Cladistia). *J. Morphol.* 268:1021–1039.

Goodrich, E. S. (1958). *Studies on the Structure and Development of Vertebrates*. New York: Dover Publications.

Graham, J. B. (1997). *Air-Breathing Fishes: Evolution, Diversity and Adaptation.* San Diego: Academic Press.

Graham, J. B. (2006). Aquatic and aerial respiration. In *The Physiology of Fishes*, 3rd ed., eds. Evans, D. H., Claiborne, J. B. Boca Raton: CRC Press, 85–117.

Graham, J. B. & Wegner, N. (2010). Breathing air in water and in air. In *Respiratory Physiology of Vertebrates: Life with and without Oxygen*, ed. Nilsson, G. E. New York: Cambridge University Press, 174–221.

Gudger, E. W. (1946). Oral breathing valves in fishes. *J. Morph* 79, 263–288.

Helfman, G. S., Collette, B. B., & Facey, D. E. (1997). *The Diversity of Fishes*. Malden, Mass: Blackwell Science.

Jollie, M. (1984). Development of the head and pectoral skeleton of *Polypterus* with a note on scales (Pisces: Actinopterygii). *J. Zool. Lond*. 204**,** 469–507.

Lechleuthner, A., Schumacher, U., Negele, R. D, & Welsch, U. (1989). Lungs of *Polypterus* and *Erpetoichthys*. *J. Morphol.* 201, 161–178.

Luther, A. (1914). Ueber die vom *N. trigeminus* versorgte Muskulatur der Ganoiden und Dipneusten. *Acta Soc. Sc. Fenn.* XLI, No. 9.

Magid, A. M. A. (1966). Breathing and function of the spiracles of *Polypterus senegalus*. *Anim. Behav.* 14, 530–533.

Müller, J. (1846). *Ueber den Bau und die Grenzen der Ganoiden, und fiber das natfirliche System der Fische*. Berlin.

Perry, S. F. (2007). Swimbladder-lung homology in basal osteichthyes revisited. In *Fish Respiration and Environment,* eds. Fernandes, M. N., Rantin, F. T., Glass, M. L., Kapoor, B. G. Enfield (NH): Science Publishers, 41–54.

Pollard, H. B. (1891). On the anatomy and phylogenetic position of *Polypterus*. *Anat. Anz. Bd*. VI.

Purser, G. L. (1926). *Calamoichthys calabaricus* (J.A. Smith). Part I. The alimentary and respiratory systems. *Trans. R. Soc.*, Edinburgh 54: 767-784.

Traquair, R. H. (1871). On the cranial osteology of *Polypterus*. *J. Anat. and Physiol. Lond.* V.