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Gills develop early in coral reef fishes: respiratory and ionoregulatory processes

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Declaration of the Contribution of Others

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Abstract

Coral reef ecosystems are among those that are most heavily impacted by anthropogenic disturbances, including climate change, overfishing, and other activities resulting in habitat loss. Consequently, studies have found that fish exposed to these disturbances respond negatively, both behaviourally and physiologically. However, these studies have primarily focused on adult fishes; whereas early life stages – the seeds of adult populations – are substantially understudied. Nonetheless, early life stages are thought to be most vulnerable to environmental stress, owing to rudimentary organs and underdeveloped physiological systems. Processes that are likely to be impacted are those related to the respiratory (oxygen and carbon dioxide exchange) and ion/osmoregulatory (ion and water balance) systems because of potential mismatch between the environmental pressures and the pace at which these systems develop.

In fish, respiration and ion/osmoregulation are known to primarily occur at the gills; however, during early life stages, before the gills form, these physiological processes are maintained through cutaneous pathways. The transition of these processes from cutaneous (i.e., skin) to branchial (i.e., gill) has been thoroughly investigated in model species, such as the rainbow trout (*Oncorhynchus mykiss*), where it is thought that gills begin functioning for ion regulation (15 days post hatch; dph) before oxygen uptake (23-28dph). This information is not known for coral reef fishes. Although, this transition is thought to occur earlier in coral reef fishes than in temperate species, like the rainbow trout, because of the influence of warm temperatures and low oxygen levels on metabolic processes. In addition, this transition is thought to be energetically costly under ideal environmental conditions, and perhaps, further exacerbated by anthropogenic stressors. Developmental deficiencies to those systems could lead to reductions in whole organism performance and ecosystem health.

The following thesis investigates gill development in coral reef fish species with the aim to determine the onset of respiratory and ionoregulatory systems. This is the knowledge base needed to better predict how anthropogenic stress could affect coral reef ecosystems. To do this, two coral reef fish species (*Acanthochromis polyacanthus* and *Amphiprion*

melanopus) were chosen. As pomacentrids, these two species belong to one of the most speciose coral reef fish families and are of high ecological importance. In addition, both are easily maintained in captivity, life cycles have been closed, genomes fully sequenced, and as a result, both are quickly becoming model laboratory species. This further highlights the importance of understanding the development of these basic but critical processes associated with respiration and ion regulation. The first experiments **(Chapter 2)** were designed to describe, in detail, the ontogeny of gill development in two coral reef fishes. I found gill tissues to form, in these two species, faster than in any other studied fish to date. Specifically, all gill structures were apparent prior to hatching. The onset of gill development may be a critical developmental window, as it is often associated with high mortality, and therefore, should be a milestone that future studies consider when investigating coral reef fish populations.

The accelerated developmental pace observed in these two coral reef fish species is perhaps related to the environmental conditions they experience (e.g., warm water temperatures during the day and low oxygen levels at night) that demand respiratory efficiency. Theoretically, the onset of gill development should coincide with increased oxygen requirements beyond what can be provided through cutaneous pathways (i.e., the oxygen hypothesis). In **chapter 3**, I tested this hypothesis by measuring the oxygen uptake of *Amphiprion melanopus* and *Acanthochromis polyacanthus* over their embryonic development. When fish start using their blood (i.e., using haemoglobin; Hb, the oxygen carrying protein found in nearly all vertebrate species) to bind oxygen from the environment and deliver it to their tissues, they are presumably using the gills to do this. If I inhibit the function of Hb (e.g., using phenylhydrazine (PHZ) to breakdown Hb), the fish is then only able to rely on cutaneous pathways to uptake oxygen. Therefore, when Hb is required to enhance oxygen transport, this should coincide with the onset of gill function for respiration. I found that, prior to hatching, embryos are able to satisfy oxygen demand via cutaneous pathways (i.e., in the absence of hemoglobin) alone. Upon hatching, cutaneous respiration was insufficient, suggesting that gills are critical for respiration after this stage. Linking these physiological findings with observations of gill development in coral reef fish obtained in **chapter 2**, my results suggest that the rapid onset of gill formation during embryogenesis is essential to meet oxygen requirements of fish larvae immediately after hatching. However,

the presence of gill tissue before it is required for branchial oxygen uptake suggests that other processes may be initiating and requiring gill formation pre-hatch.

The ionoregulatory hypothesis suggest that cutaneous processes for ion/osmoregulatory balance become insufficient before those for respiration because transporters are localised within specialised cells. Therefore, surface area to volume ratios (SA/V) will limit ion exchange before limiting respiration. In **chapter 4**, I used immunohistochemistry to observe the primary enzymatic ion exchanger, $Na^+ K^+ ATPase$ (NKA), on the skin, yolk-sac, and the gills in developing embryos of two coral reef fish species to determine when ion regulation processes begin. I found NKA to first appear on the skin and yolk-sac at 1-2 days post fertilization (dpf) and on the gill filaments at 3-5 dpf. My study is the first to provide evidence that coral reef fishes use NKA external to the gills, i.e., through cutaneous pathways. Additionally, these fishes show an accelerated developmental pace of ion regulation, with the earliest appearance of NKA on the skin and the gills in any species investigated to date. The early appearance of ionoregulatory cells on the gills before the formation of lamellae and before the onset of branchial oxygen uptake suggests that ion regulation is the primary driver underpinning gill development in these fishes. My findings support the ionoregulatory hypothesis.

The first critical developmental window for coral reef fishes is prior to hatching, when gills begin to form. Therefore, anthropogenic stressors or other environmental perturbations that could disrupt gill development pre-hatch may preclude healthy adult populations and coral reef ecosystems. Here, I emphasize that embryonic life stages must be considered when investigating the impacts of anthropogenic stressors on coral reef fishes. Processes performed by the gills, if impeded, could have negative cascading effects throughout development and into adulthood. Overall, understanding the structure and function of gills and how and when they develop is essential to understanding fish ecology and evolution, but can also be key to effectively managing fish populations in a changing world.

Contents

List of Tables

List of Figures

List of Appendices

1 General Introduction

Fishes represent 50% of all described vertebrates (IUCN, 2014) and inhabit an immense array of aquatic ecosystems found on Earth (Hurley et al., 2007). The ecological and evolutionary success of fishes is attributed to a range of structural and physiological adaptations that help them survive and reproduce in the aquatic environment (Randall et al., 2014, Rummer et al., 2013, Evans et al., 2005). Nevertheless, fishes also share a common suite of adaptations that help them meet core demands for oxygen (O_2) and nutrients and thus maintain critical metabolic processes (Evans, 1998). Gills are among the most common of these adaptations (occurring to some extent in all fishes) and are the primary structures that allow fishes to overcome the challenge of obtaining O_2 and removing metabolic waste within their aquatic setting (Evans et al., 2005). Consequently, understanding the structure and function of gills is essential to our understanding of fish ecology, evolution, and management, and can help to predict the response of fish communities to a changing environment (Hess et al., 2017, Bowden et al., 2014).

1.1 Gill Structure and Function

In aquatic environments, obtaining O_2 is a much greater challenge than in air. This is because the viscosity of water is 35-100 times greater than that of air and 800-1000 times denser; therefore, diffusion rates of molecules in water can be up to 300,000 times slower than in air and the availability O_2 substantially lower in water (i.e., 0-10mL/L in water vs. 210mL/L in air; Schmidt-Nielsen, 1997, Nilsson, 2010). As a result, gills must be highly specialised to enhance the process of extracting O_2 and removing waste in the aquatic environment. Gills are positioned in direct contact with the external environment to allow water to be consistently drawn across the epithelium and close to the heart with a series of blood vessels passing through them (Hughes and Morgan, 1973). Structurally, the gills of ray-finned fishes (teleosts) consists of four arches with two rows of filaments and a large number of plate-like structures known as lamellae extending from each side of each filament (Figure 1-1; Evans et al., 2005, Hughes, 1984). The lamellae are often as thin as 1-2 cell

layers and are thought to be the primary site for O_2 uptake, as they have an immense surface area and potentially short diffusion distances (Hughes and Morgan, 1973, Rombough and Moroz, 1997). In general, fast swimming marine fishes such as mackerel or tuna display lamellae densities of 26-31 lamellae per millimeter of filament, whereas slow swimmers such as European sea bass possess only 21-26 lamellae per millimeter of filament (Hughes and Morgan, 1973, Tarallo et al., 2016, Gray, 1954). However, tropical coral reef fishes have been found to possess lamellae densities between 28-34 lamellae per millimeter of filament (Bowden et al., 2014). Although coral reef fishes are not considered as highly active species, they may display lamellae densities equal to active species because they exhibit high oxygen demands associated with their environmental conditions (e.g., warm water) in which they reside. Indeed, gill morphology can be altered to respond to changing environmental conditions and thus meet the core demands of the fish (Sollid et al., 2003, Sollid and Nilsson, 2006).

The internal structure and physiology of gills is also highly adapted to facilitate O_2 extraction. Once O_2 comes in contact with the lamellae, it diffuses into the bloodstream. The flow of water and blood occurs in opposing directions, as it enters and leaves the lamellae, creating a counter-current flow (Randall and Daxboeck, 1984, Hughes and Morgan, 1973). This enhances the diffusion of O_2 into the blood, since blood is always in contact with water of a higher O_2 partial pressure (pO_2; Evans et al., 2005). Indeed, counter-current flow is so efficient that it enables 80-90% of the water's O_2 to be extracted (Figure 1-1; Evans et al., 2005, Schmidt-Nielsen, 1997). By comparison, *Homo sapiens* – at best – can extract only 25% of the available oxygen from a single breath. As in most other vertebrates, fish blood also contains haemoglobin (Hb; highly specialised protein) that enhances the maximal amount of O_2 carried in the blood by close to 30-fold when compared to O_2 transport without Hb (Nikinmaa, 2011). For most organisms, a decrease in blood pH decreases the affinity of Hb for O_2, which is responsible for releasing O_2 to the tissues (Hilpert et al., 1963). In teleosts, however, decreases in blood pH not only decrease the affinity of Hb for O_2, but also lowers the carrying capacity of Hb for O_2 (Root, 1931), thus generating perhaps the most efficient O_2 transport system currently known in all extant vertebrates (Rummer and Brauner, 2015, Rummer et al., 2013, Randall et al., 2014).
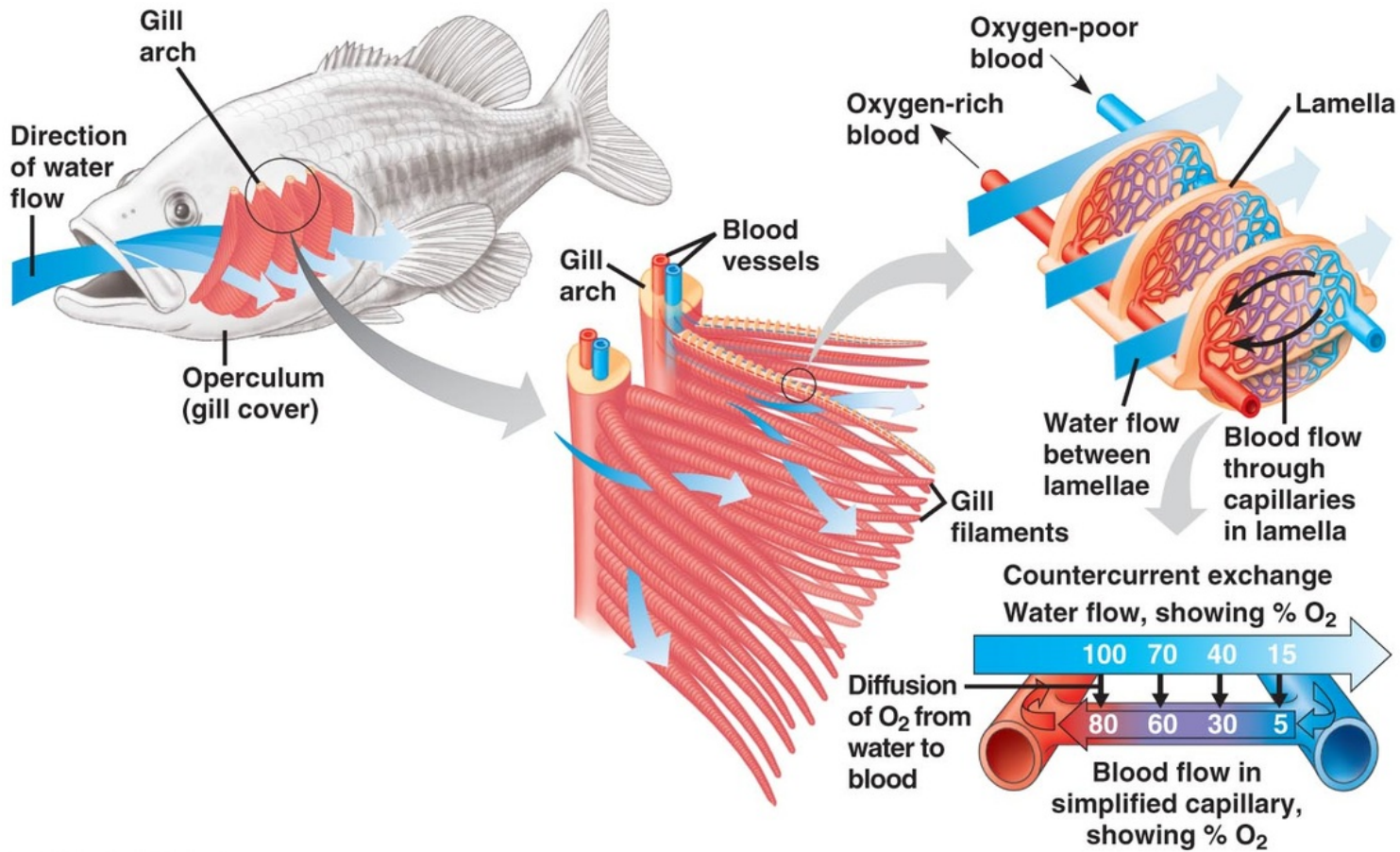
Figure 1-1: Schematic of fish gills and their function, demonstrating the role of multi-scale morphologies on gas/solute exchange (sourced from (Reece et al., 2016)

Beyond the extraction of O_2, gills are also the primary site for ion/osmoregulation, the processes of maintaining the balance of water and salt concentrations (Evans, 2008, Evans et al., 2005). Ion/osmoregulation in fish is influenced by their external environment; to maintain their internal osmotic concentrations between 250-500 milliosmoles (mOsmol/kg), freshwater fishes absorb salts through their gills, whereas marine fishes excrete salts through their gills. In freshwater environments, the osmotic concentration (0.1 mOsmol/kg) is several times lower than the internal osmotic concentration of the fish, therefore, freshwater fishes are continually at risk of being ion deficient. Freshwater fishes cope with these conditions by limiting the amount of water ingested and regularly removing large amounts of diluted urine. In contrast, in the marine environment, the osmotic concentration of the surrounding water is typically 2-4 times greater than the internal osmotic concentration of a marine teleost. Therefore, marine teleosts are continually subjected to the loss of water due to the high salt content of their environment (Evans, 1998). Marine teleost fishes counteract this process by up-taking high loads of seawater to replenish water levels; however, a by-product of this is that salt concentrations increase. To counteract the accumulation of salt, fish use ionocytes, generally occurring on gill filaments, to regulate Na^+ and Cl^- ion concentrations (Foskett and Scheffey, 1982). These cells utilise three major ion-transporters that regulate the flow of salt ions across the gill membrane (Hiroi et al., 2008, Hirose et al., 2003) and work concomitantly to remove excess salts.

Finally, fishes utilise their gills to maintain internal pH and remove nitrogenous waste (Randall, 2005). Internal pH buffering in teleost fishes is extremely efficient, where it occurs to some degree in the tissues and in the blood (Heisler, 1984). However, H^+ ions still build up during metabolic processes and, therefore, transporters are required to remove them and further protect internal pH (Evans et al., 2005). In marine fishes, our understanding of acid/base balance at the gill is limited; however, it is thought that Na^+/H^+ antiporter exchangers within ionocytes are responsible. The role of gills in the removal of nitrogenous waste is also not clear. However, in marine fishes, Na^+/H^+ exchangers are also thought to be used, perhaps occurring in conjunction with acid/base regulation (Evans, 1998).

1.2 Gill Development – The Two Hypotheses

Gill structure and function during early life stages of fishes has been relatively unexplored compared to that of adult stages. In the early 1990s, studies started focusing more

on the onset of gill function during early development and the external or internal drivers of gill formation. Today, researchers consider two hypotheses to explain what function is driving gill development in teleost fishes. The first hypothesis was proposed by Krogh (1941), who suggested that gills develop to meet the O_2 demands of a developing fish. The more recent, second hypothesis suggests that ion regulation is thought to be the first function of gills. This was proposed because ion regulation becomes limited before O_2 uptake and, therefore, ion/osmoregulation is the driving force underpinning gill development (Li et al., 1995). Understanding whether gills develop primarily for respiratory or ionoregulatory purposes is important for projecting how larval fishes may respond to environmental change, highlighting the potential causes of developmental deficiencies, and revealing the role of gills in vertebrate evolution (Rombough, 2007).

The oxygen hypothesis: During early development, fish embryos and larvae maintain O_2 supplies by absorbing O_2 across the skin and yolk-sac epithelium and directly into the tissues or plasma, a process known as cutaneous respiration (Feder and Burggren, 1985). Before gills form in early larval stages, the epithelium surface area is large enough to satisfy O_2 demand; however, this method of O_2 uptake is not sustainable because the fish's surface area to volume ratio (SA/V) declines as the fish grows. According to the oxygen hypothesis, the increasing biomass of tissues relative to the area of skin available for O_2 to be obtained gradually becomes insufficient to meet the O_2 requirements, thus necessitating the development of gills (Krogh, 1941). Theoretically, gills develop primarily to function as a site for O_2 uptake, otherwise limited O_2 supply would quickly lead to asxphyation and death as adult stages progress (Krogh, 1941).

The ionoregulatory hypothesis: Similar to O_2 uptake, ion regulation is maintained through cutaneous pathways prior to gills forming. In several species, ionocytes have been found on the yolk-sac and skin epithelium, and are suggested to perform the same ionoregulatory tasks as branchial ionocytes (Hiroi and McCormick, 2012, Kaneko et al., 2002). As described above for O_2 uptake, the available surface area for ionocytes to perform ionoregulatory functions become limited with growth. Nevertheless, the ionoregulatory hypothesis suggests that cutaneous ion regulation becomes limited earlier than O_2 uptake. This is because the function of ionocytes to transport ions requires their apical membrane to be exposed to the exterior environment while their basal membrane be in direct contact to the blood sinus (Alderdice, 1988). As the skin thickens, however, the chance of ionocytes

reaching both the exterior environment and the blood significantly reduces, limiting the fish's capacity to transport ions across the skin. Consequently, ion regulation becomes limited before O_2 uptake, thereby acting as the primary driver underpinning gill development.

The ion regulation hypothesis was first suggested when Li et al. (1995) found ionocytes on gill structures formed prior to lamellae in developing tilapia (*Oreochromis mossambicus*). Since then, numerous studies have also found ionocytes to transition to the gills before or simultaneously to lamellae formation (Table 1-1; Rombough, 2007). However, these data are based on morphological evidence only, and do not involve direct measurements of O_2 uptake and ion regulation. Recently, *in situ* experiments have measured O_2, sodium (Na^+), and ammonia (NH_4^+) movements in developing rainbow trout larvae (*Oncorhynchus mykiss*). Estimates for both branchial (gill) and cutaneous (skin) movements were obtained by separating the anterior (primarily branchial respiration) and posterior (primarily cutaneous respiration) sections of the fish. The point at which 50% of activity was cutaneous and 50% was branchial was set as the transitioning point depicting when the gill was required for either function. At 15 days post hatch (dph), Na^+ and NH_4 movements transitioned to the gills; whereas, O_2 uptake transitioned later, around 23-28 dph (Fu et al., 2010, Zimmer et al., 2014). These *in situ* observations provide critical evidence in support of the ionoregulatory hypothesis.

While *in situ* experiments reveal exact trends of movements between the cutaneous and branchial pathways, they have only been used with salmonids (chinook salmon, Atlantic salmon and rainbow trout; Rombough and Ure, 1991, Wells and Pinder, 1996, Rombough, 1998, Fu et al., 2010). This is because larval salmonids are relatively large, and *in situ* experimental manipulation is more easily achieved (Brauner and Rombough, 2012). However, comparing *in situ* data to morphological data collected on salmonids validates the accuracy of morphological observations. For instance, morphological data in rainbow trout revealed sharp declines of extra-branchial ionocyte abundance at 48 days post fertilization (dpf), which corresponds to the timing of when Na^+ exchange transitioned to the gills, at 45-48 dpf (Fu et al., 2010, Rombough, 2007, Brauner and Rombough, 2012). Perhaps, morphological data can be useful and potentially be a key tool for analysing gill development in smaller fishes.

Previous research investigating gill development has focussed primarily on freshwater species (Rombough, 2007). Freshwater and marine fishes exhibit opposing mechanisms for ion/osmoregulation, with Na^+ absorption in freshwater species versus Na^+ excretion in saltwater species (Evans, 1998, Evans et al., 2005). This may drive different developmental patterns in marine taxa if ion excretion and absorption become limiting at different rates. Furthermore, tropical marine fishes are relatively understudied, and tropical coral reef fishes are yet to be investigated. Warmer temperatures can raise metabolic rates and lower the availability of oxygen, potentially leading to a greater demand for O_2 uptake during early developmental stages (Angilletta et al., 2002, Clarke and Johnston, 1999). Thus, elucidating the role of O_2 uptake and ion regulation in gill formation in tropical marine species remains a key challenge.

Table 1-1 Timing of mitochondria rich cells on filaments and lamellae development in teleost fishes. All timings are displayed as days post fertilization (dpf) to standardise comparisons.

Common Name	Species Name	Temperature (°C)	Gill Structures (dpf)		References
			Ionocytes on Filaments	Lamellae	
tilapia	*Oreochromis mossambicus*	27	7	7	(Li et al., 1995)
American shad	*Alosa sapidissima*	20	8	8	(Gao et al., 2016)
senegal sole	*Solea senegalensis*	20	6.5	9.7	(Padros et al., 2011)
flatfish brill	*Scophthalmus rhombus*	15	15	12	(Hachero-Cruzado et al., 2009)
zebrafish	*Danio rerio*	28	5-7	12-14	(Rombough, 2002)
rainbow trout	*Oncorhynchus mykiss*	8	30	36	(Rombough, 1999)
ayu	*Plecoglossus altivelis*	17-20	31.5	46.5	(Hwang, 1990)

1.3 Physiological Characteristics of Tropical Coral Reef Fishes

Coral reefs are one of the most biologically diverse ecosystems on Earth. They occupy less than 0.1% of the world's oceans, yet they support up to 25% of all known marine fishes and provide high social and economic importance (Moberg and Folke, 1999). Coral reefs are also among the most threatened ecosystems worldwide due to increasing human stressors such as climate change, over-fishing, and activities resulting in habitat loss (Halpern et al., 2008). For instance, reefs in recent years have experienced major mass bleaching events linked to anthropogenic global warming (Hughes et al., 2003, Hughes et al., 2017). Studies on adult reef fishes have shown negative responses to elevated temperatures (Laubenstein et al., 2018, Rummer et al., 2014, Scott et al., 2017, Scott et al., 2018, Donelson et al., 2012, Donelson et al., 2011). A temperature increase of +3°C has been found to cause declines in aerobic scope (i.e., difference between maximum and resting aerobic metabolism) and reductions in gill health (Rodgers et al., 2018). However, majority of these studies have focused on adult and juvenile life stages, where only a few studies have investigated larval and embryonic life stages – the seeds for adult populations.

Larval and embryonic life stages of coral reef fishes are known to be highly vulnerable, experiencing natural mortality rates of up to 60% per day (Goatley and Bellwood, 2016). During these early developmental periods, physiological processes are thought to be rudimentary and are not yet fully developed (e.g., hypoplasia; Falk-Petersen, 2005). Therefore, exposure to acute and/or drastic environmental change may be detrimental to these life stages, as their internal systems may fail to respond. For instance, being ectothermic organisms, a fish's internal temperature mirrors their environmental temperature; therefore, if the environmental temperature increases, so will the internal temperature of the fish. As a result, metabolic rates will increase, resulting in elevated O_2 demand (Clarke, 2003, Angilletta et al., 2002). The capacity to meet O_2 demand at higher temperatures could potentially be more problematic during early life stages in coral reef fishes, as the fish may lack complete functioning respiratory tissues (i.e., gills) and would, therefore, be unlikely to obtain satisfactory amounts of oxygen. Thus, the timing of gill development in coral reef fishes will be critical for their survival, particularly during periods of high stress.

Despite the importance of gills for fitness and survival, no studies to date have addressed the timing of gill development in coral reef fishes. Larvae of several coral reef fishes have exhibited advanced swimming abilities, high O_2 consumption rates, and the capacity to withstand low O_2 levels that are known to be lethal to other fishes (Stobutzki and Bellwood, 1994, Nilsson et al., 2007, Nilsson, 2010). Based on these exceptional physiological characteristics, it may be hypothesized that gills form and function earlier in development relative to other fishes. Additionally, coral reef ecosystems, due to their warm water temperatures and low O_2 levels at night, may further drive the demand for gills early in development (Nilsson and Ostlund-Nilsson, 2004, Almeida-Val et al., 2006). Indeed, previous respiratory studies on coral reef fishes suggest they are already living at or beyond their thermal optimal temperature (Rummer et al., 2014). Here, I specifically hypothesize that gill development in coral reef fishes may occur at an accelerated pace, earlier than what is known in other investigated fishes. Accelerated development of gills may arise because the thermal environment and physiological characteristics of larval coral reef fishes necessitate a greater supply of O_2 early in development, and thus, provide support for the oxygen hypothesis. Alternatively, however, the ion/osmoregulatory properties of gills in coral reef fishes may play a more immediate role in gill development as shown in previously studied fishes, and thereby support the ionoregulatory hypothesis.

1.4 Thesis Outline

In the following thesis, I aimed to examine the developmental sequence of gills in two tropical coral reef fishes, specifically to understand the transition from cutaneous to branchial sites for O_2 uptake and ion regulation. Two coral reef fish species, the spiny chromis (*Acanthochromis polyacanthus)* and the cinnamon clownfish (*Amphiprion melanopus),* were chosen as study species since they belong to one of the most speciose coral reef fish families and are of ecological importance (Cowman, 2014, Ceccarelli et al., 2001). Additionally, these fishes are easily transitioned into captive/aquaria settings where their lifecycles have been closed. I had three main objectives to gain insight into the gill development of coral reef fishes. My first objective was to use visual and histological techniques to determine when gills begin to form during ontogeny and to compare the rate of gill formation in coral reef fishes with other described taxa. The second objective of this project was to determine when O_2 uptake transitions from cutaneous pathways to branchial pathways during development. By measuring O_2 uptake rates in embryos with inhibited Hb function, I can determine when

gill respiration and Hb activity begins. My third objective was to identify ionocytes using immunohistochemistry as a proxy for ion regulation. Using this protocol, I was able to determine, for the first time, if coral reef fishes use extra-branchial ionocytes, and when these cells transition to the gills. Overall, by comparing all three data chapters, I was able to determine which process is the primary driver underpinning gill formation and function in coral reef fishes. This investigation provides insight into the onset of critical physiological processes underlying their survival, and potentially demonstrating developmental patterns that are unique to coral reef fishes.

1.5 Conclusion

The results of this thesis produced a developmental timeline depicting a critical developmental window of coral reef fishes that can be used in future studies. For instance, studies investigating how coral reef fishes will respond to anthropogenic stressors can target this early developmental period and begin to identify potential developmental deficiencies. Alterations to gill development during early life stages may impede the succession into adult life stages, ultimately upscaling to community and ecosystem levels. Therefore, understanding the developmental onset of gill structures and functions in early life stages is key to improving the resolution of future coral reef fish populations as well as coral reef fisheries and, overall, to help maintain healthy ecosystems.

2 Rapid development of gill morphology in tropical coral reef fishes

Chapters 2-4 have been prepared for publication in a peer-reviewed, scientific journal.

2.1 Introduction

Gills have been attributed as one of the most important organs associated with the success of aquatic organisms. In fishes, this owes to gills serving as the primary site for some of the most vital physiological processes, such as oxygen (O_2) uptake, ion/osmoregulation, acid/base regulation and ammonia excretion (Evans et al., 2005). On top of these crucial processes, gills also provide a secondary site for hormonal control, immune defence, and food capture (Evans, 1998). Undeniably, gills are providing a multitude of services to the organism, including those that are of crucial importance to survival and this is because gills are a structurally-adapted organ. For instance, gills are positioned in close proximity to the heart, attain a series of circulatory vessels, and are continually exposed to the external environment where water consistently draws across the epithelium (Hughes and Morgan, 1973). Structurally, gills consist of four arches with two rows of filaments and lamellae extending from each side of the filament (Figure 1-1; Evans et al., 2005, Hughes, 1984), allowing surface area to increase allometrically with growth (Hughes and Morgan, 1973, Rombough and Moroz, 1997). Together, these adaptations facilitate efficient exchanging, sourcing, and delivery of materials to and from the cells as well as between the internal and external environments.

During early life stages, before gills form, O_2 uptake, ion/osmoregulation, acid/base regulation and ammonia excretion are maintained through cutaneous pathways (i.e., across

the skin; Feder and Burggren, 1985, Glover et al., 2013). The skin of developing fishes is extremely thin and exhibits a relatively large surface area, allowing the movement of materials to transfer via simple diffusion between the internal and external environments. However, as a fish grows, the surface area to volume ratio (SA/V) declines, ultimately becoming insufficient to sustain these physiological processes. Theoretically, this is when these processes are thought to transition to the gills (Rombough and Moroz, 1997). Determining when these processes first function at the gills is not uniform and is most likely to be influenced by the rate at when they become limited and their importance for survival. Temperature – the pacemaker of growth – is likely to have one of the largest influences on when these processes become limited and drive gill development (Angilletta et al., 2002). Indeed, cold water species, such as the rainbow trout (*Oncorhynchus mykiss*), begin gill development at 17 days post fertilization (dpf), and their lamellae form later at 36 dpf (Rombough, 1999); whereas, warm water species, such as the zebrafish (*Danio rerio*), begin gill development at 3 dpf and their lamellae form at 12 dpf (Rombough, 2002). However, other factors alongside differences in temperature may also play significant roles in gill development.

Salinity, ambient O_2 levels, and lifestyle are all known to influence the rate of gill-mediated processes, and therefore, during ontogeny, may dictate when gills develop by increasing the demand for such processes. For example, in general, the demand for ion/osmoregulatory balance in freshwater fishes is greater than in marine fishes, while the respiratory demand in marine fishes is greater than in freshwater fishes. This is due to differences in salinity and ambient O_2 levels between freshwater and marine environments (Schmidt-Nielsen, 1997, Nilsson, 2010). Additionally, lifestyles are also likely to be influential, where more active fishes have greater demand for O_2 uptake than inactive fishes (Blank et al., 2007). Therefore, the timing of when gills are required would be a result of the accumulated pressures generated from the environmental factors and lifestyles they experience during early development. However, teasing apart these factors to determine the most demanding remains a challenge. This is because majority of fishes that have been investigated so far are freshwater and/or temperate water species (Rombough, 2007); diversity among environmental factors and lifestyles is lacking. For example, fish that reside in tropical, marine environments are substantially understudied, although present an interesting view. In theory, tropical fishes are faced with large constraints (i.e., high metabolic rates and lower ambient oxygen levels) that are demanding to gill-mediated

processes, such as tropical marine fishes. In fact, a substantial group of tropical marine fishes, i.e., coral reef fishes, are yet to be investigated.

Coral reef fishes present an interesting study taxon because of their challenging environmental conditions and lifestyles during early life stages. Coral reef fishes reside in warm waters that endure nightly periods of low oxygen, and upon hatching, undergo a pelagic larval duration (PLD; Leis and McCormick, 2002, Nilsson and Ostlund-Nilsson, 2004). Larval coral reef fishes during their PLD are known to exhibit advanced swimming abilities and high O_2 uptake rates (Stobutzki and Bellwood, 1997, Stobutzki and Bellwood, 1994, Nilsson et al., 2007). Together, these factors would demand a sufficient respiratory organ early in development. Here, I set out to document the sequential pattern of gill formation in coral reef fishes. Specifically, I aim to document the development of key morphological structures underlying gill function, compare different paces of gill development in tropical coral reef fishes with that of other taxa, and discuss the potential causes and consequences of these differences. This study addresses a key knowledge gap by producing a timeline of gill development that can be used when investigating the impacts of anthropogenic stressors on gill tissue, development, and function.

2.2 Methods

James Cook University Animal Ethics Committee approved the following experiments under ethics protocol A2407.

Study species and Rearing Protocols

Embryos from two coral reef fish species, *Acanthochromis polyacanthus* and *Amphiprion melanopus* (Family: Pomacentridae), were used in this study. Embryos were sourced from established breeding pairs of both species being held in 60L flow-through (UV-filtered seawater) aquaria facilities at the Marine and Aquaculture Research Facility (MARFU) at James Cook University (JCU). Breeding pairs were exposed to ambient photoperiods, and water temperature was maintained at 28.5 ± 1°C. Breeding pairs were provided with half a terracotta pot for shelter and embryo deposition. Fish were fed twice daily (morning and evening) with INVE Aquaculture NRD 1.2mm food pellets (ProAqua Pty Ltd, Queensland, Australia) to satiation. Upon morning feeding, pots were subsequently checked for egg deposition. If present, embryos from both species were sampled every

morning from the day of deposition until one day prior to hatching (n= 6 per age). For *Am. melanopus,* hatching occurred at 7 days post fertilization (dpf), and for *Ac. polyacanthus,* hatching occurred at 10 dpf.

Histological techniques; sampling and assay

Embryos were removed from pots and examined under a dissecting microscope (Olympus SZX7 Stereomicroscope System), observing the onset of the heartbeat, blood flow (including colour) and the presences of gill structures. The embryos were micrographed using Olympus SC50 digital camera. Embryos were euthanised by being placed into ice cold water, fixed in Bouin's solution for 12-24hrs, and transferred into 70% ethanol until histological preparation. Six samples per age were used for histology.

To prepare samples for histological assays, all embryos were dechorinated (removal of chorion or egg capsule) using Dumont Super fine-tips forceps under a dissecting microscope (Olympus SZX7 Stereomicroscope System), micrographed using an Olympus SC50 digital camera for total length, and placed into cassettes. Total length was measured in ImageJ (v. 1.51s, National Institutes of Healthy, Rockville, MD, USA). Samples underwent a series of ethanol and wax infiltrations (Shandon Southern Duplex Processor BS5) and were then embedded in paraffin blocks (Shandon Histocentre 3, Thermo Electron Corporation). Samples were then sectioned at 5μm thin using a microtome and further stained using Mayer's Hematoxylin and Eosin's Young. Slides were then examined using a compound microscope (Olympus BX41 Microscope), and images were taken using an Olympus SC50 digital camera, ensuring that entire embryos were in the field of view (*Ac. polyacanthus* was taken at 50x and *Am. melanopus* was taken at 100x). Morphological features, including number of arches, rows of filament, and presence of lamellae were recorded from micrographs.

2.3 Results

In *Acanthochromis polyacanthus,* at 1 day post fertilization (dpf), the yolk-sac and rudimental body and head regions were evident. At 2 and 3 dpf, the heart began to beat and red blood circulation occurred, respectively (Figure 2-1). Gill arches appeared as early as

3dpf but were evident by 4dpf in all investigated embryos (Figure 2-1). After gill arches formed, gill filaments were evident, appearing between 4 and 5 dpf, but most commonly at 5 dpf (Figure 2-1; Figure 2-2) after which, lamellae formation occurred. Lamellae were visualised as early as 5 dpf, most commonly appearing at 6-7 dpf (Figure 2-2), but as late as 8 dpf. At 8 and 9 dpf, lamellae proliferated across all filaments (Figure 2-1). Hatching generally occurred at 10 dpf ($\pm$ 1 dpf).

In *Amphiprion melanopus*, the yolk-sac, rudimental body and head regions were evident at 1 dpf. A beating heart and red blood circulation commenced at 2 and 3 dpf, respectively (Figure 2-1). In the majority of *Am. melanopus* embryos examined, gill arches formed at 3 dpf (Figure 2-2); however, in one embryo, gill arches formed at 4 dpf (Figure 2-1). Gill filaments appeared predominantly at 4 dpf, but by 5 dpf, and lamellae formed soon after at 5 dpf (Figure 2-2). Although, one *Am. melanopus* embryo was found to develop lamellae at 6dpf (Figure 2-1). Hatching occurred at 7 dpf ($\pm$ 1 dpf).
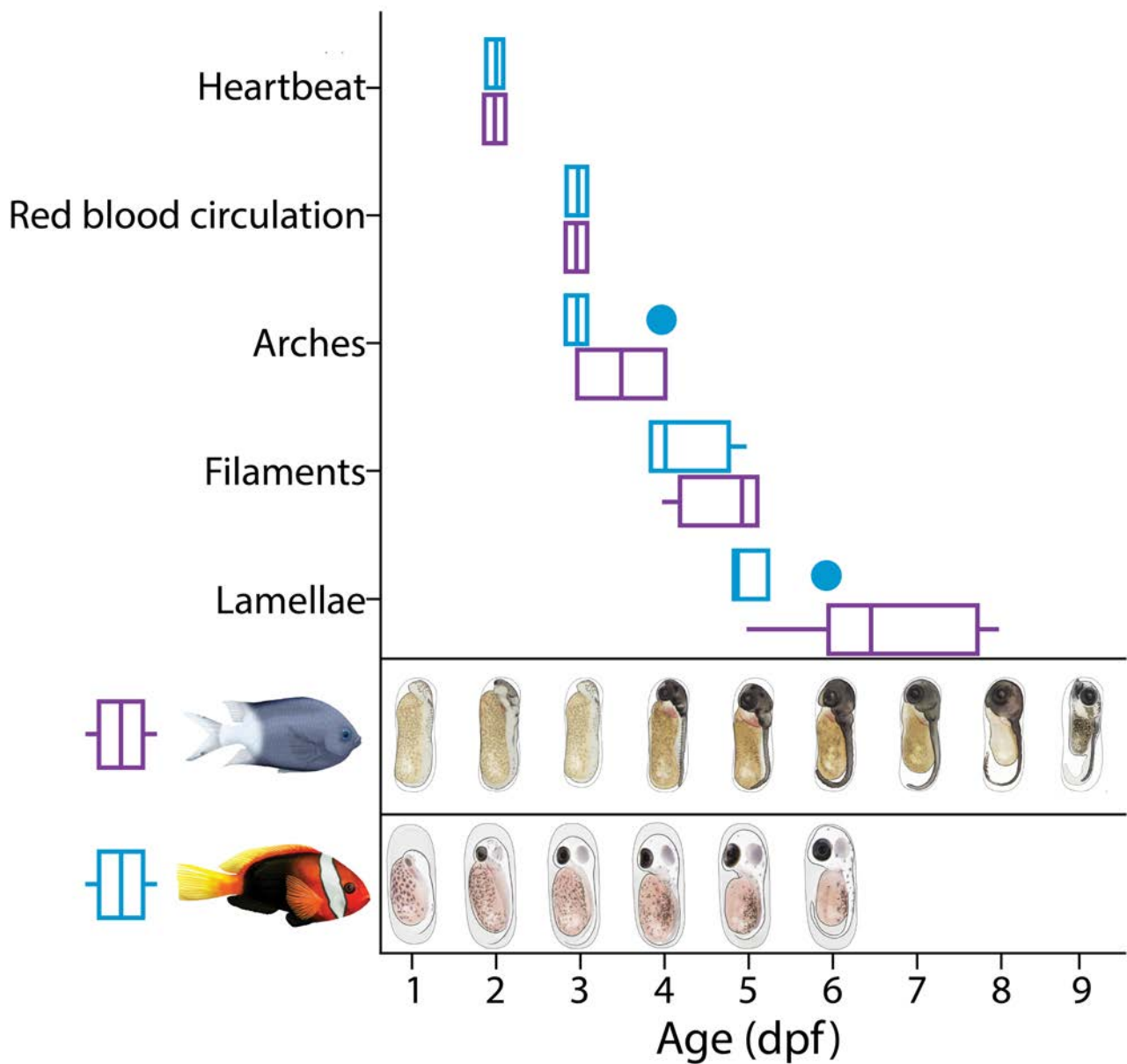
Figure 2-1: Sequential appearance of functions and tissues in developing embryos. Horizontal boxplots represent mean, upper, and lower quartiles, and dots represent outliers. Drawings represent developmental growth throughout embryology. *Ac. polyacanthus* is in purple and *Am. melanopus* is in blue.
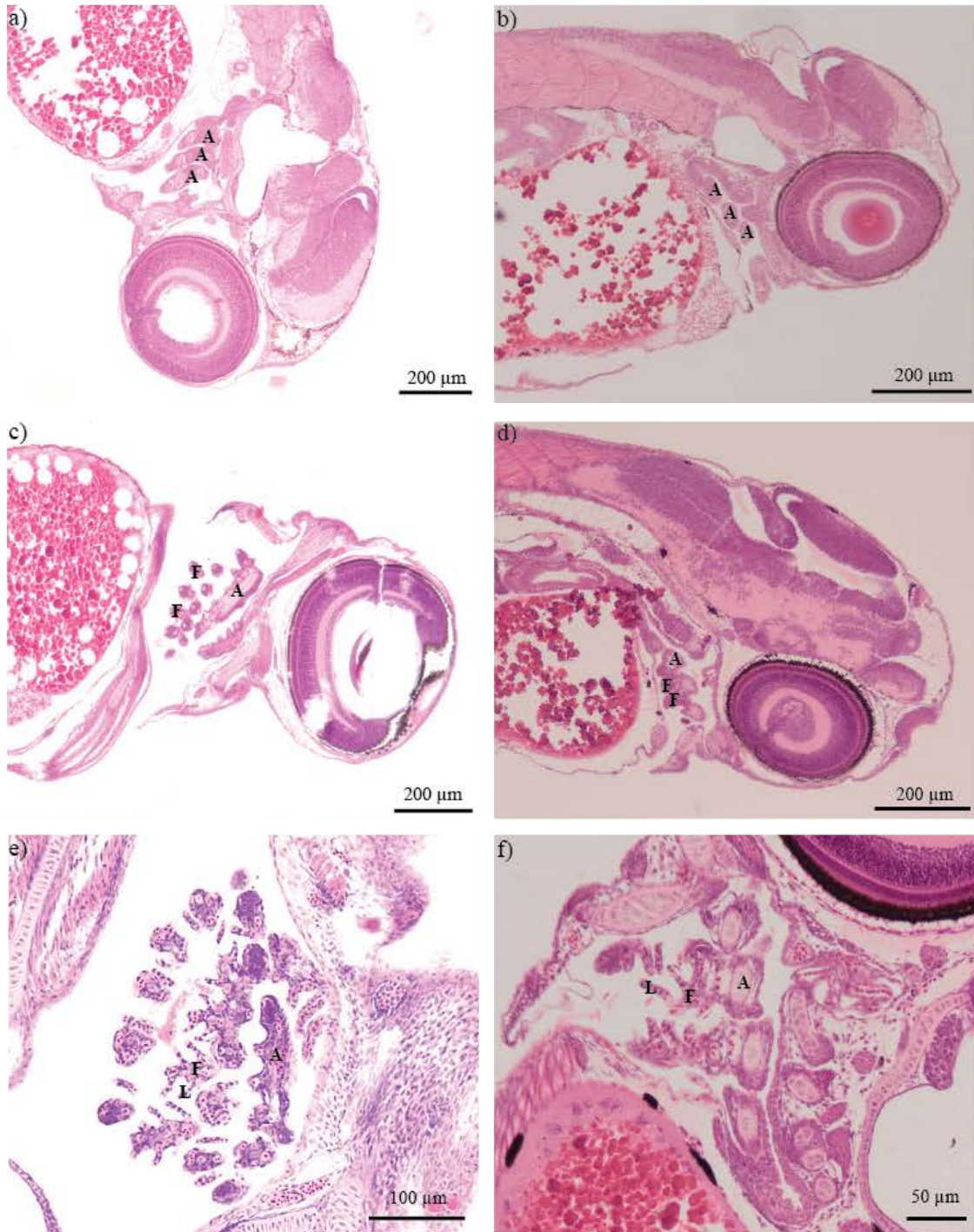
Figure 2-2: Histological micrographs (H&E stain) of gill development in: *Ac. polyacanthus* embryos at **a)** 4 days post fertilization (dpf), **c)** 5 dpf, and **e)** 7dpf; and *Am. melanopus* embryos at **b)** 3 dpf, **d)** 5 dpf, and **f)** 6dpf. **A**, arches; **F**, filament; **L**, lamellae.

2.4 Discussion

Like other fish species, the appearance of key gill structures in our two studied coral reef fish taxa occurred in a specific sequence (Olson, 2002). Gill arches appeared first at 3 dpf, providing the hard bony structures that house the afferent and efferent branchial arteries; the first entry/exit point of blood flow between the organ and the body (Hughes, 1984, Olson, 2002). The next structures to form were the gill filaments at 4 dpf, the first gill structure that allows for substantial increases in SA/V that cannot occur on the skin (Lefevre et al., 2017, Rombough and Moroz, 1997). The filaments also allow for the extension of afferent and efferent branchial arteries, thus enhancing blood flow throughout the gills (Hughes, 1984, Olson, 2002). Lastly, the lamellae first emerged between 5 and 7 dpf, and this is thought to be a crucial development for aerobic activities because lamellae are the primary site for O_2 uptake in later life stages (Hughes and Morgan, 1973). Indeed, the formation of lamellae provides a critical stepping-stone in the transition of O_2 uptake from the skin to the gills.

Interestingly, the timing it took for each gill structure to form in these two species occurred at an accelerated pace. Gill structures were termed complete when lamellae were present, which occurred at 7 dpf in *Ac. polyacanthus* and 5 dpf in *Am. melanopus.* This represents the earliest known appearances of lamellae in any fish species studied thus far (Table 2-1). For example, lamellae formation appears at 8 dpf in American shad (Gao et al., 2016), 9.7 dpf in Senegal sole (Padros et al., 2011), 12 dpf in flatfish brill (Hachero-Cruzado et al., 2009), 36 dpf in rainbow trout (Rombough, 1999), and 46.5 dpf in Ayu (Hwang, 1990). However, comparing our two study species with the previously listed species is not necessarily valid, as it is well-known that growth and development are highly influenced by temperature (Clarke, 2003). In contrast to these previously studied species, my two study species reside on coral reefs that are tropical ecosystems experiencing warm water temperatures and relatively low O_2 levels at night (Nilsson and Ostlund-Nilsson, 2004, Almeida-Val et al., 2006). Together, these factors exacerbate the challenge of maintaining core demands. Specifically, elevated temperatures are known to raise metabolic rates in ectothermic organisms, such as fish, thus creating higher O_2 demand, which would be particularly challenging during nightly periods of low ambient O_2 (Clarke, 2003). However, developmental rates in the two tropical coral reef fishes examined here were still faster than other investigated warm-water taxa. Indeed, at temperatures between $28 \pm 1°C$, lamellae

begin developing at 7 dpf in tilapia (*Oreochromis mossambicus)* (Li et al., 1995) and at 12-14 dpf in the zebrafish (*Danio rerio*; Rombough, 2002). Therefore, other factors beyond temperature and O_2 levels are playing major roles in the accelerated gill development observed in these two coral reef fish species, and perhaps others.

Table 2-1 Timing of gill structure development in teleost fishes. All timings are displayed as days post fertilization (dpf) to standardise comparisons.

Common Name	Species Name	Temperature (°C)	Gill Structures (dpf)			References
			Arches	Filaments	Lamellae	
cinnamon clownfish	*Amphiprion melanopus*	28	3	4	5	This study
spiny chromis	*Acanthochromis polyacanthus*	28	3	4	7	This study
tilapia	*Oreochromis mossambicus*	27			7	(Li et al., 1995)
American shad	*Alosa sapidissima*	20	4	5	8	(Gao et al., 2016)
senegal sole	*Solea senegalensis*	20	6.5	6.5	9.7	(Padros et al., 2011)
flatfish brill	*Scophthalmus rhombus*	15	6	7	12	(Hachero-Cruzado et al., 2009)
zebrafish	*Danio rerio*	28	3		12-14	(Rombough, 2002)
rainbow trout	*Oncorhynchus mykiss*	8	17-20	27	36	(Rombough, 1999)
ayu	*Plecoglossus altivelis*	17-20	6.5	6.5	46.5	(Hwang, 1990)

Physiological traits that are unique to larval coral reef fishes could be influencing gill development rates. The larvae of several coral reef fish have been found to exhibit enhanced oxygen uptake rates ($\dot{M}O_2$; e.g., 5250 mg kg^{-1} h^{-1}) that are currently the highest $\dot{M}O_2$ recorded in ectothermic vertebrates. Some coral reef fishes have been found to exhibit the fastest sustained swimming speeds (50 body lengths per second; BL sec^{-1}) of all other studied fishes for their body size (Stobutzki and Bellwood, 1994, Fisher et al., 2005). Furthermore, upon settlement (i.e., transition from pelagic to reef association), some coral reef fish species are among the most hypoxia tolerant studied to date (Nilsson et al., 2007). To sustain these physiological traits, it is presumed a large respiratory surface area and functioning haemoglobin (Hb) are required (Sollid and Nilsson, 2006, Sollid et al., 2005). Therefore, the accumulative pressures larval coral reef fishes face, on top of the challenging environmental conditions, may be the underlying drivers underpinning accelerated gill development. However, other physiological processes performed at the gills such as ion regulation also need to be considered.

Ion regulation and other processes may play a more immediate role in gill development than O_2 uptake. The onset of gill structures occurs in sequence of ionoregulatory tissue (i.e., filaments) before respiratory (i.e., lamellae) and therefore could suggest ionoregulatory processes begin performing at the gills first. Additionally, during early life stages, high loads of metabolic wastes, in particular lethal nitrogenous waste, would accumulate due to cellular processes associated with high growth rates (e.g., organo- and morphogenesis) and yolk-sac consumption (Wright et al., 1995, Dhiyebi et al., 2013, Zimmer, 2014). Therefore, developing coral reef fishes would require established methods to remove these wastes before they reach toxic levels (Randall and Tsui, 2002). In fact, proficient metabolic waste removal processes would be required before enhanced O_2 uptake processes, as these high rates of growth and yolk-sac consumption occur during the embryonic period and thus before oxygen-demanding traits. While I am not yet able to determine whether O_2 uptake, ionoregulatory processes, or other influences such as reproduction influence gill development the most in coral reef fishes, the above findings provide a framework on which to further explore the driving forces for gill development.

The two coral reef species examined here exhibited similar developmental patterns; although, *Am. melanopus* completed gill development (i.e., based on lamellae formation)

earlier than *Ac. polyacanthus*. The reason for this developmental difference may originate from the different life histories of the two species. For instance, *Am. melanopus*, like most other coral reef fishes, undergoes a pelagic larval phase from hatch to settlement. During this phase, this species resides in the pelagic zone, where it disperses and metamorphoses before returning to the reef habitat. In contrast, *Ac. polyacanthus* lacks a pelagic phase, whereby upon hatch the larvae reside by the parents, receiving some parental care (Leis and McCormick, 2002). While these two different reproductive strategies do not appear to have a major influence on the pattern of development (i.e., both species hatching with equipped gills), the pelagic phase of *Am. melanopus* could necessitate the earlier appearance of gill tissue and faster development of gill-mediated processes to meet the challenging conditions (e.g., evading predators, sourcing food, and finding suitable habitat) in the open ocean; although, immediate life on the reef could also be argued as a more challenging environment.

Conclusion

Overall, this study found gill structures to form early in coral reef fishes, when compared to other species of fishes that have been studied to date. In these two species of coral reef fishes, all gill structures were present prior to hatching. These results emphasise that coral reef fishes' embryonic phase may be a critical developmental window, as gills perform important physiological processes such as respiration and ion regulation, which are key processes underpinning fish survival. If stressors interfere with the onset or ability of gill-mediated processes, development and growth of the individual may be impaired, perhaps leading to a failure of the individual to reach later life stages. Nevertheless, the driving force behind this early, accelerated gill development is yet to be examined, and a detailed account of gill function during early development is key to our understanding of this critical development window.

3 Oxygen consumption rates in larval reef fish require early gill development

Chapters 2-4 have been prepared for publication in a peer-reviewed, scientific journal.

3.1 Introduction

Oxygen (O_2) is a fundamental requirement for nearly all living organisms (Nilsson, 2010, Schulte, 2015). The amount of O_2 required for aerobic respiration is dependent on an individual's metabolic rate (Baker et al., 2010). In fishes, metabolic rates during early life stages are known to be exceptionally high to sustain growth and development (Pedersen, 1997). Larval coral reef fishes, in particular, have the highest oxygen uptake rates ($\dot{M}O_2$) of all studied ectothermic vertebrates, with rates of up to 5,250 mg O_2 kg^{-1} h^{-1} (Nilsson et al., 2007). Such high $\dot{M}O_2$ may be elicited by high rates of growth and swimming in reef fishes in conjunction with warmer waters (Stobutzki and Bellwood, 1997). Moreover, gill development in coral reef fishes occurs at comparatively exceptional rates (see Chapter 2) during embryonic development, but gill development is thought to be extremely energetically expensive (Jones and Randall, 1979). Therefore, it may be predicted that embryos of coral reef fishes exhibit increased $\dot{M}O_2$ in comparison to other fishes. A key question remains. How do coral reef fishes meet such high O_2 demand during their early life stages?

Before the formation of gills in fishes, O_2 requirements are fulfilled by obtaining O_2 across cutaneous pathways (e.g., skin), a process known as cutaneous respiration (Feder and Burggren, 1985). Cutaneous pathways however, as a fish grows, are unable to sufficiently maintain a fish's aerobic scope due to the declining surface area to volume ratio (SA/V) of the fish's body (Rombough and Moroz, 1997), and at this point, respiratory processes will transition to the gills. In addition to declining SA/V, the fish's skin thickens with growth, increasing the distance O_2 has to travel to enter the internal environment and consequently,

decreases diffusion efficiency. Understanding the transition between cutaneous and branchial respiratory pathways is crucial, as it is a milestone in the progression of growth and development in fishes. Indeed, if this period of gill development is interfered with, the ability for the fish to meet O_2 demands may be affected, causing negative impacts for all aerobic activities, such as growth, foraging, predator evasion, and metamorphosis. This may cause a succession of negative impacts, where young fish are unable to reach adulthood, therefore altering population or fishery dynamics.

Here, I set out to determine when O_2 uptake transitions from cutaneous to branchial pathways in two tropical coral reef fishes and how this transition is linked with high O_2 demands associated with early development. To do this, I obtained estimates of $\dot{M}O_2$ and quantified the contribution of cutaneous respiration throughout early ontogeny by inhibiting Hb action. While the O_2 carrying role of Hb is critical for gill respiration, cutaneous respiration can continue in its absence (Jacob et al., 2002, Pelster and Burggren, 1996). The time at which cutaneous respiration becomes limiting to the fish in the absence of Hb may therefore, be used to pinpoint the transition between cutaneous and branchial respiration. In combination with results obtained in chapter two (see page 15), I can begin to identify whether the onset of branchial O_2 uptake is a major driver underpinning early gill development.

3.2 Methods

James Cook University Animal Ethics Committee approved the following experiments under ethics protocol A2407.

Study species and Rearing Protocols

Embryos from two coral reef fish species, *Acanthochromis polyacanthus* and *Amphiprion melanopus* (*Family:* Pomacentridae), were used in this study. Embryos were sourced from established breeding pairs of both species being held in 60L flow-through (UV-filtered seawater) aquaria facilities at the Marine and Aquaculture Research Facility (MARFU) at James Cook University (JCU). Breeding pairs were exposed to ambient photoperiods, and water temperature was maintained at 28.5 ± 1°C. Breeding pairs were provided with half a terracotta pot for shelter and embryo deposition. Fish were fed twice daily (morning and evening) with INVE Aquaculture NRD 1.2mm food pellets (ProAqua Pty

Ltd, Queensland, Australia) to satiation. Upon morning feeding, pots were checked for egg deposition.

Respirometry experiments; exposure, setup and trials

Hb inaction and cutaneous-only respiration was induced by treating individuals with phenylhydrazine (PHZ). $\dot{M}O_2$ was subsequently measured in embryos from 1 dpf until 100% mortality occurred in individuals exposed to PHZ. Embryos were removed from terracotta pots on the morning of the trial, transferred into two closed exposure tanks, and suspended in mesh (1.5mm x 1.5mm; 4-8 individuals per exposure tank). A continuous stream of air was directed across the mesh-enclosed embryos, where they remained for a duration of 3-hours. Of the two tanks, one was exposed to PHZ (2mg/L) and the other remained as control (Jacob et al., 2002, Pelster and Burggren, 1996). Exposure time was determined through pilot studies, where embryos exposed to PHZ were examined under a dissecting microscope (Olympus SZX7 Stereomicroscope System) for the absence of red blood, indicating Hb was destroyed (Appendix 1; Pelster and Burggren, 1996). Tanks were cleaned using bleach after every exposure period.

To measure $\dot{M}O_2$, embryos (n= >15 per age) were individually placed into a well (150 μl, painted black), sealed with a rubber stopper, and submerged in a temperature-controlled flow-through water bath (Figure 3-1; modified from Pasparakis et al. (2016)). Each well was equipped with an O_2 sensor spot (REDFLASH dye) connected to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) by a 2m fibre optic cable. The Oxygen sensor was calibrated following the Pyroscience user manual. The chambers were assembled as described for experimental trials; however, for 100% oxygen calibration, chambers were placed open into a clean seawater bath with multiple functioning air stones, and for 0% oxygen calibration, chambers were filled with oxygen depleted freshwater using sodium sulphite (Na_2SO_3). Once the oxygen sensor reading stabilized, the calibration was set and saved. Temperature compensated O_2 levels (mg L^{-1}) were measured consistently (2 s^{-1}) for one hour for later analysis to determine the embryos' $\dot{M}O_2$. Additionally, dissolved O_2 concentrations were measured for 10 min prior to and following each trial to correct for background microbial respiration (refer to Rummer et al. 2016). Each well was tested for O_2 permeability before experiments commenced by measuring O_2 levels in deoxygenated water

for 2 hours (exceeding experimental period). Following each trial, embryos were patted dry and weighed using a microbalance.

For analysis, $\dot{M}O_2$ above 80% dissolved O_2 were analysed and obtained through using linear least squares regression in LabChart version 7.2.5 (ADInstruments, Colorado Springs, CO, USA; Pasparakis et al., 2016, Peck and Moyano, 2016). $\dot{M}O_2$ were measured in three-minute intervals, plotted against time with volume of wells, background respiration compensated, and corrected for body mass (Rummer et al., 2016, Norin and Clark, 2016). The highest $\dot{M}O_2$ was used for statistical analysis, representing a stressed $\dot{M}O_2$. The highest rate generally occurred within the first slope calculated; however, all occurred within the first 9 minutes of the measurement period (Norin and Clark, 2016). A stressed $\dot{M}O_2$ was used rather than resting, since resting $\dot{M}O_2$ may mask the effects of PHZ. In other words, the amount of O_2 obtained through cutaneous pathways may meet the requirements of a resting embryo and therefore, differences between treatments would be undetectable. However, obtaining an estimate of increased $\dot{M}O_2$ should reveal differences between treatments if the embryos were relying on branchial respiration to obtain oxygen.

Statistical Analyses

All statistical analyses were performed in R version 1.1.453. Linear mixed-effects models (LME) were used to observe changes in $\dot{M}O_2$ as a function of embryo developmental time and PHZ exposure. $\dot{M}O_2$ was log transformed in both species to meet normality, and the best fit model was determined by comparisons using Akaike information criterion (AIC). Models were also visually assessed through Q-Q plots and residual vs fitted plots to assess for normality and equal variances. For the LME model, logged $\dot{M}O_2$, temperature, age, treatment, and fish body mass were used as fixed effects, and clutch was included as a random effect. Temperature and mass were both mean-centered to improve interpretation. All models with significant differences were used in a Tukey's multiple comparison test (glht, corrects *p*-values) to find where significant differences occur between ages.
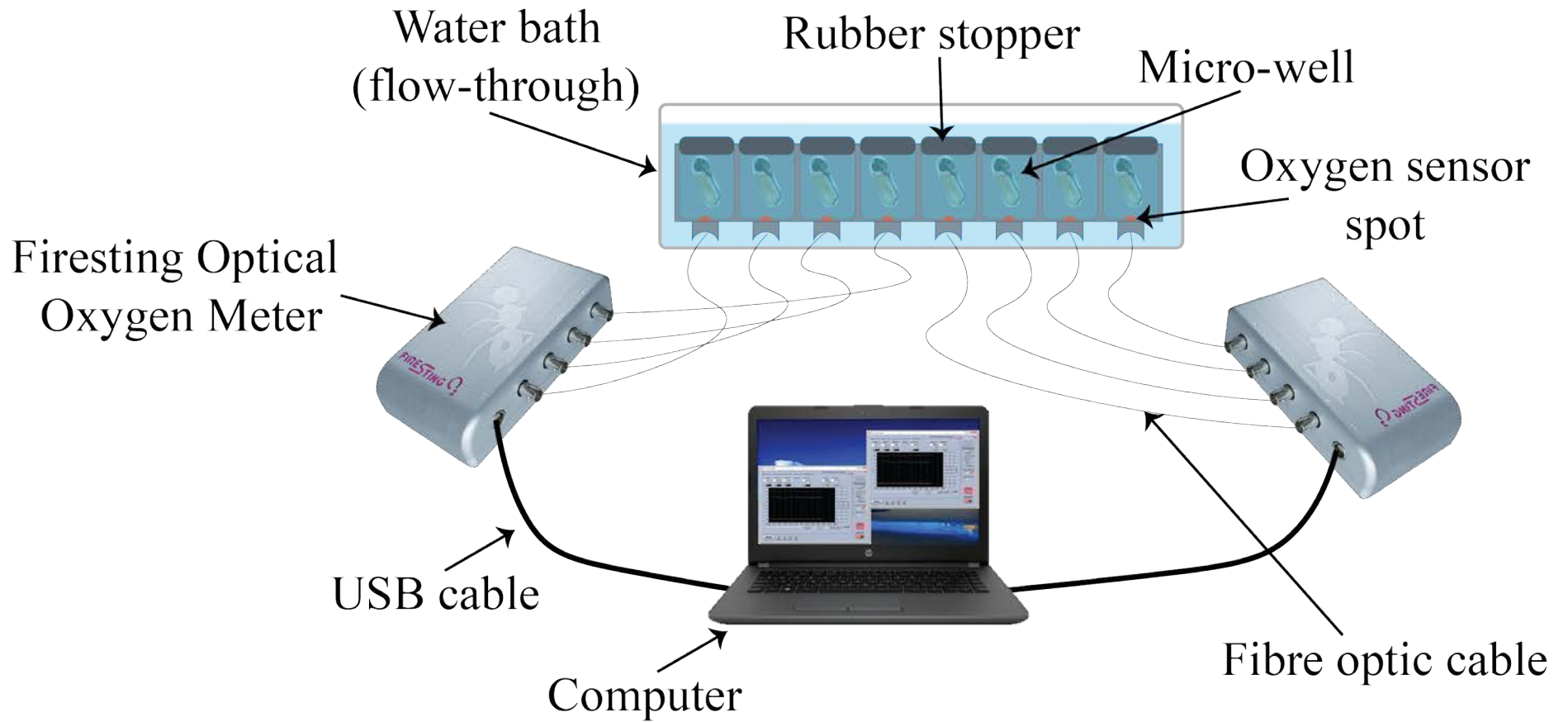
Figure 3-1: Micro-respirometry system. Modified from Pasparakis et al. (2016).

3.3 Results

$\dot{M}O_2$ increased throughout development in both species as the fish grew larger. Changes in $\dot{M}O_2$ across development were significant in both species; *Ac. polyacanthus* ($F_{8,344}$=31.3808, *p*=<0.0001; Figure 3-2; Appendix 2) and *Am. melanopus* ($F_{5,233}$=6.135, *p*=<0.0001; Figure 3-2, Appendix 2). Hereafter, when gills started to form, I found $\dot{M}O_2$ to remain stable, where no spike in $\dot{M}O_2$ corresponded with the completion of gill formation. Specifically, in *Ac. polyacanthus*, $\dot{M}O_2$ significantly increased with age, where $\dot{M}O_2$ in 1 day post fertilization (dpf) embryos was significantly lower than in 2-9 dpf embryos, the $\dot{M}O_2$ of 2 dpf embryos was significantly lower than the $\dot{M}O_2$ of 4-9 dpf embryos, and the $\dot{M}O_2$ of 3 dpf embryos was significantly lower than in 4-9 dpf embryos (Appendix 3). The $\dot{M}O_2$ values at 4 dpf through to 9 dpf were not significantly different despite the onset of lamellae at 7 dpf (Appendix 3). For *Am. melanopus*, $\dot{M}O_2$ was only found to be significantly different at 2 dpf and 5 dpf (Appendix 4).

In *Ac. polyacanthus* embryos, there was a significant interaction between treatment and age on $\dot{M}O_2$ ($F_{8,344}$=2.1628, *p*=0.0298; Figure 3-2; Appendix 2); at 4 dpf $\dot{M}O_2$ of PHZ treated embryos were slightly smaller than $\dot{M}O_2$ in control embryos (*z*=-3.497, *p*=0.0491; Figure 3-2; Appendix 5). No significant differences occurred in any other age. Overall, PHZ had a minimal impact on $\dot{M}O_2$ at almost all ages observed in the analysis and did not affect $\dot{M}O_2$ reached prior to hatching. No significant interaction between treatment and age on $\dot{M}O_2$ was found in *Am. melanopus* ($F_{5,233}$=1.474, *p*=0.1992; Figure 3-2; Appendix 2). However, in both species, 100% mortality occurred in fishes exposed to PHZ at 1day post hatch (1dph).
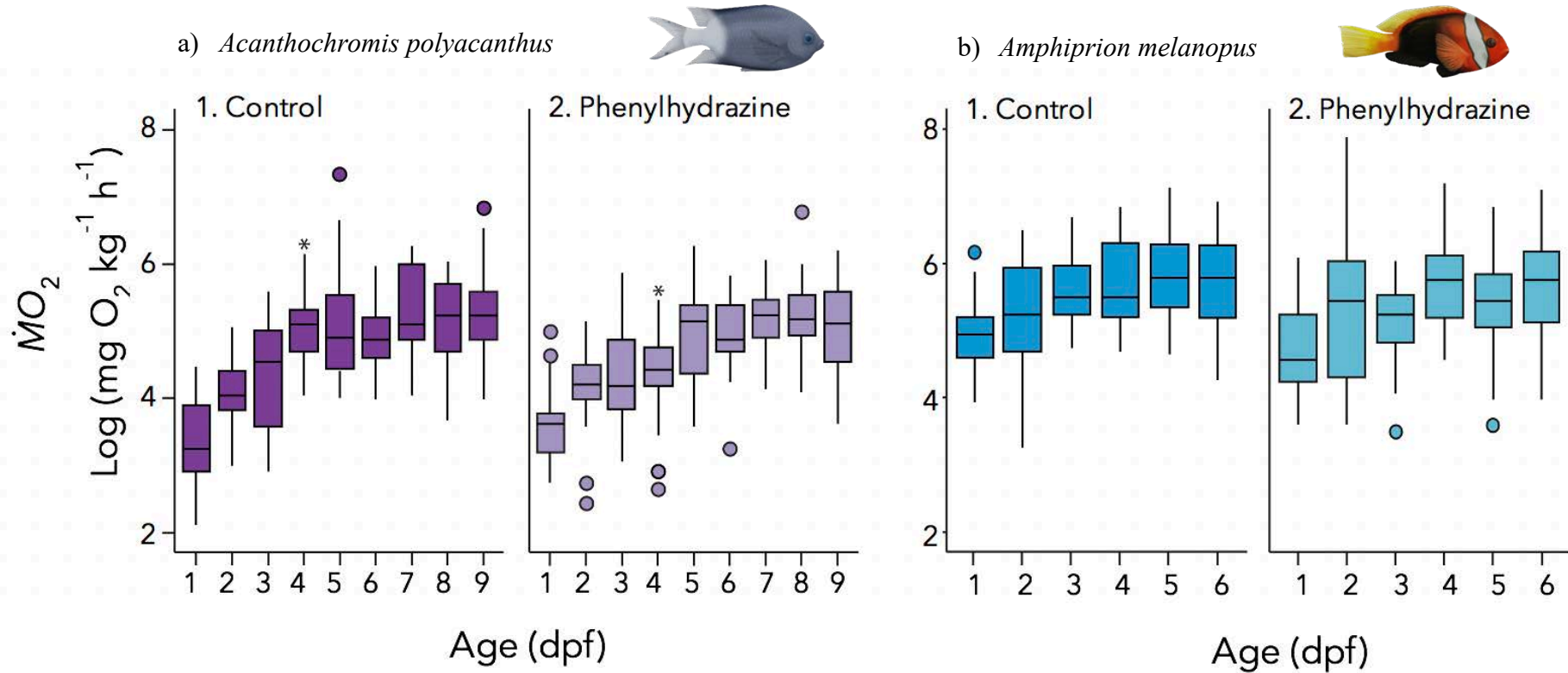
Figure 3-2: Oxygen uptake throughout embryonic development in: **a)** *Acanthochromis polyacanthus*, and **b)** *Amphiprion melanopus,* separated by treatments: **1.** Control and **2.** Phenylhydrazine exposed (quantifying cutaneous respiration). Boxplots represent median, upper and lower quartiles. All species at 1dph when exposed to phenylhydrazine resulted in 100% mortality. Asterisks indicate significant differences between treatments when compared against equal age at $\alpha = 0.05$.

3.4 Discussion

Oxygen uptake during embryonic development is met through cutaneous pathways in the two coral reef fish species studied here. Estimates of $\dot{M}O_2$ remained similar between total and cutaneous measurements, as revealed by similar $\dot{M}O_2$ values over time in fishes with inhibited Hb (cutaneous pathways only) and uninhibited Hb (cutaneous and branchial pathways). Prior to gill structure formation, and in particular, lamellae (3-6 dpf), when cutaneous pathways were the only source of O_2 uptake, inhibiting Hb did not lower $\dot{M}O_2$, confirming that cutaneous pathways were not affected by the presence or absence of Hb in this study. Similarly, in other species, Hb was not found to play any major roles in cutaneous respiration, where $\dot{M}O_2$ with or without Hb was not significantly different from each other (Jacob et al., 2002, Pelster and Burggren, 1996). Moreover, after the formation of lamellae, inhibiting Hb continued to have little effect on $\dot{M}O_2$, providing evidence that gills were unlikely aiding in O_2 uptake processes during embryogenic development. These results suggest that cutaneous respiration plays a critical role in early development and is sufficient to satisfy O_2 requirements throughout the fish's embryonic development.

It is apparent that cutaneous pathways are able to meet the O_2 requirements during embryonic development; however, upon hatch, my results show cutaneous pathways become fundamentally insufficient. It appears that the majority of O_2 uptake transitions from cutaneous pathways to the gills in newly hatched coral reef fishes, where 100% mortality was found in newly hatched fish with inhibited Hb, i.e., larvae reliant on only cutaneous respiration. Hb is known to play important roles in O_2 delivery to the tissues and to enhance $\dot{M}O_2$ at the gills (Nikinmaa, 2011). Therefore, these results suggest that if O_2 is diffusing across the gills in a similar fashion to cutaneous respiration, i.e., without Hb, gills alone are unable to satisfy O_2 demands upon hatching. Therefore, upon hatching, developing coral reef fishes are requiring gills coupled with functional Hb to meet these increased O_2 demands. The transition of O_2 uptake from cutaneous to branchial pathways appears to be a drastic advancement occurring immediately between changes in life stages (i.e., embryo to larvae) and extremely early in development.

Interestingly, the transition between cutaneous and branchial pathways for O_2 uptake in this study occurs earlier than any other investigated teleost species. For example, in a

warm water species, the zebrafish, O_2 uptake does not begin to occur at the gills until 14 dpf, approximately 11.5 days after hatching (Rombough, 2002). In another tropical fish species, tilapia, it is unknown when gills become dependent; however, lamellae do not begin to form until 7 dpf or 3 dph; assuming that O_2 uptake would transition sometime after that (Hwang et al., 1994, Ayson et al., 1994). Nevertheless, these two previously studied warm water species do not compare to the rapid rates of gill development and onset of branchial O_2 uptake in the two current coral reef fish species, and perhaps early onset of branchial respiration is related to differences in O_2 demands associated with changes from being a sessile embryo to an active newly hatch.

The early onset of branchial respiration in tropical coral reef fishes is possibly linked to the increased O_2 demands that occurs post-hatch, where larvae are involved in more frequent and enhanced aerobic activities. For example, coral reef fishes during their larval phase are known to exhibit advanced swimming abilities, begin exogenous feeding, while avoiding predators and undergoing major organ changes or metamorphosis (Nilsson, 2007, Stobutzki and Bellwood, 1994, Fisher et al., 2005, Donelson et al., 2012, Green and McCormick, 2001). The onset of these activities could be the result of drastic increases in $\dot{M}O_2$ that occur upon hatch. For example, estimates of maximum $\dot{M}O_2$ in 1 dph *Ac. polycanthus* larvae were measured at >6000 mg O_2 kg^{-1} h^{-1} (unpublished data, Downie et al), that is ~30 times higher than $\dot{M}O_2$ in pre-hatch embryos measured in this study. This drastic increase in $\dot{M}O_2$ that occurs between pre- and post-hatch stages supports my finding that O_2 uptake transitions to the gills upon hatching. However, gills begin forming before they are required to enhance O_2 uptake, suggesting that other gill-mediated processes may play a more immediate role.

The gill apparatus during early life stages are known to be energetically expensive and are suggested to be associated with the cost of development and other gill-mediating processes. In several salmonids, gills have been found to consume O_2 during early development (Fu et al., 2010, Rombough, 1998, Rombough and Moroz, 1997, Rombough and Ure, 1991, Wells and Pinder, 1996). For example, in rainbow trout larvae, partial pressure of oxygen (PO_2) measured in the skin surface was significantly higher than the PO_2 within blood vessels that are associated with the gills, and at later larval stage, PO_2 in the efferent branchial artery leading away from the gills was significantly lower than the vitelline

vein leading to the gills, suggesting that gills were consuming rather than sourcing O_2 (Rombough, 1992). Our data show minimal O_2 is being sourced at the gills during embryogenic development and like salmonids, gills at this point could be consuming O_2 to cover the energetic cost of gill tissue growth. Additionally, in several species, other gill-mediated processes such as ion regulation have also been found to perform at the gills prior to O_2 uptake (Rombough, 2007, Brauner and Rombough, 2012) that, in addition to development, could also be requiring oxygen. This may be the case for the two species present in this study.

Conclusion

This study found that inhibiting O_2 uptake at the gills during a critical development window (i.e., hatching) was fatal to coral reef fish species, emphasizing the importance of gill development for survival. This is the earliest known time for teleost fishes to begin relying on gills for O_2 uptake and is suggested to be associated with oxygen-demanding activities that larval coral reef fishes undergo. Nevertheless, in these two coral reef fish species, the gill structures become fully developed during the embryo phase and interestingly, before they become the primary site for O_2 uptake. A more immediate role could be performed by the gills before O_2 uptake, such as ionoregulatory processes. Therefore, this study further highlights the need for investigation into other roles performed by the gills to identify critical processes that underpin gill formation, as they may play larger roles to the success and survival of the individual.

4 Accelerated onset of ion regulation in early life stages of coral reef fishes

Chapters 2-4 have been prepared for publication in a peer-reviewed, scientific journal.

4.1 Introduction

Adult teleost fishes use their gills, kidneys and intestines for ion/osmoregulation, acid-base regulation and ammonia excretion (Evans et al., 2005). After development, the gills are known to be the primary site for maintaining these physiological processes. While acid-base regulation and ammonia excretion within the gills remain relatively similar between species in adult life stages, ion/osmoregulatory often differ, and in most cases, these processes change in response to the external environment. For example, in freshwater teleost fishes, ion/osmoregulatory processes mainly consist of obtaining sodium (Na^+) and other ions, where, without this process, freshwater fishes would suffer from ion loss. In contrast, marine teleost fishes are face with the opposite situation and that is being susceptible to dehydration. This is because marine fishes reside in environments that are approximately 1000 milliosmols (mOsmol/kg), which is 2-4 times greater than their internal osmotic concentration (250-500 mOsmol/kg). Marine fishes therefore, are continually subjected to water loss due to the natural gradient formed by these differences (Evans, 1998), but counteract this process by up-taking high loads of seawater to replenish water levels. Consequently, marine fishes therefore, attain excessive amounts of salts but possess sufficient ion/osmoregulatory processes to remove these excess ion loads.

Generally, fishes use ion regulating cells often referred to as ionocytes (used here on), chloride cells, or mitochondria-rich cells to exchange salts (Foskett and Scheffey, 1982). In marine fishes, ionocytes are generally found along the gill filaments, at the base of lamellae,

and possess high amounts of mitochondria and three major ion-transporting proteins; $Na^+ K^+$ ATPase (NKA), $Na^+ K^+ 2CL^-$ cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR; Hiroi et al., 2008, Hirose et al., 2003). Specifically, NKA pulls two potassium (K^+) ions into the ionocyte, while removing three Na^+ ions in exchange. These three Na^+ ions are removed via 'leaky tight junctions' within the paracellular space by electrical gradients. The NKCC cotransporter uses the negative gradient produced by NKA to remove three Na^+ ions and allows two chloride (Cl^-) ions to enter the ionocytes, as well as one Na^+ and one K^+ ion. The two Cl^- ions are then removed through the apical-located chloride channel that is analogous to the CFTR (McCormick et al., 2009, Hiroi and McCormick, 2012, Evans et al., 2005). Overall, these three proteins work concomitantly to remove excess salt loads (Figure 4-1).
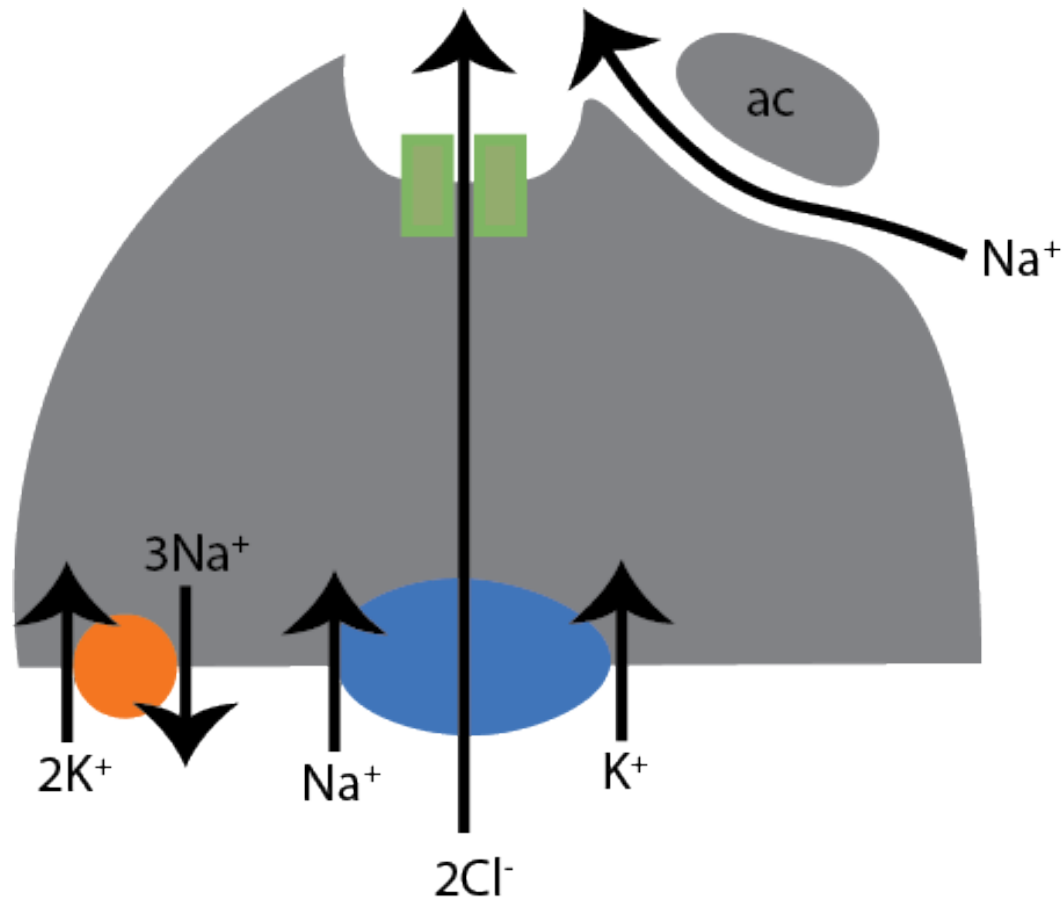
Figure 4-1: Representing NaCl excretion in a saltwater branchial ionocyte; NKA (orange) and NKCC (blue) is shown on the basolateral localization and the CFTR (Green) located apically. The intercellular space shown between the accessory cell (ac) and the ionocyte represents the movement of Na^+ through the 'leaky tight junction'. Modified from Hiroi et al (2008).

During early fish development, before gills form, ion/osmoregulation is supported through cutaneous pathways, where ionocytes have been found to form on the yolk-sac and skin of fish embryos and larvae (Alderdice, 1988, Ayson et al., 1994, Hwang, 1989, Kaneko et al., 2002). Ionocytes on the skin have been shown to be structurally different to ionocytes on the gills (Kaneko et al., 2002); however, they are thought to perform homogenous tasks to branchial ionocytes, as all three major ion-transporting proteins present in branchial ionocytes have been found within cutaneous ionocytes (Hiroi and McCormick, 2012, Hiroi et al., 2005). Therefore, fishes form cutaneous ionocytes to maintain ion/osmoregulation during early development before branchial ionocytes form; however, maintaining ion/osmoregulation across cutaneous pathways becomes insufficient with increasing fish size. At this point, ionocytes will transition to the gills. This change in pathways generally occurs immediately or soon after gills form (refer to Table 1-1 & Table 2-1). Additionally, changes in ionocyte morphology have been found to occur alongside substantial increases in branchial ionocytes, perhaps pinpointing when cutaneous ion/osmoregulation becomes limited (Hiroi et al., 1998, Rombough, 1999, Varsamos et al., 2002, Fu et al., 2010). This suggests that morphological observations may be a useful tool to estimate when ionoregulatory mechanisms become limited and begin primarily performing at the gills. Nonetheless, the shift of ionoregulatory activity between extra-branchial ionocytes (found on skin and yolk-sac) and the gills identifies a key milestone in developing fishes. In fact, this transition has been found to occur in several other fish species before O_2 uptake transitions to the gills (i.e., supporting the ionoregulatory hypothesis).

The ionoregulatory hypothesis for gill development suggests that limitations to cutaneous ion regulation during development are driving gill formation. Increasing evidence supports the ionoregulatory hypothesis across multiple species (Rombough, 2007); however, tropical coral reef fishes had not yet been investigated until this study. Because I have found tropical coral reef fishes to have rapid gill development when compared to other teleost fishes and that they begin oxygen uptake processes early in ontogeny (chapters two and three), it follows that understanding when the gills begin performing ionoregulatory roles is of crucial importance. By investigating the transition of ionoregulatory processes from the skin to the gills, I can pinpoint whether this occurs before or after oxygen uptake processes, therefore supporting or refuting the ionoregulatory hypothesis. In the following chapter, my aims were to describe the transition between cutaneous and branchial ion regulation and search for the

first evidence of extra-branchial ionocytes in coral reef fishes. Understanding the primary driving force for gill development is crucial for understanding early life stages in fishes and critical developmental windows that are suggested to be highly vulnerable to environmental change.

4.2 Methods

James Cook University Animal Ethics Committee approved the following experiments under ethics protocol A2407.

Study species and Rearing Protocols

Embryos from two coral reef fish species, *Acanthochromis polyacanthus* and *Amphiprion melanopus* (Family: Pomacentridae), were used in this study. Embryos were sourced from established breeding pairs of both species being held in 60L flow-through (UV-filtered seawater) aquaria facilities at the Marine and Aquaculture Research Facility (MARFU) at James Cook University (JCU). Breeding pairs were exposed to ambient photoperiods and water temperature was maintained at $28.5 \pm 1°C$. Breeding pairs were provided with half a terracotta pot for shelter and embryo deposition. Fish were fed twice daily (morning and evening) with INVE Aquaculture NRD 1.2mm food pellets (ProAqua Pty Ltd, Queensland, Australia) to satiation. Upon morning feeding, pots were subsequently checked for egg deposition. If present, embryos from both species (across minimum 3 breeding pairs) were sampled from the day of deposition until one day prior to hatching (n= 6 per age). For *Ac. polyacanthus* hatching occurred at 10 dpf*,* and for *Am. melanopus* hatching occurred at 7 dpf.

Immunocytochemistry; preparation, staining and visualising

Upon collection, embryos were euthanised and fixed in 4% paraformaldehyde for 12-24hrs at room temperature before being transferred into 70% ethanol and stored at $2°C$ until further analysis. Embryos were dechorinated (removal of chorion or egg capsule) using Dumont Super fine-tips forceps under a dissecting microscope (Olympus SZX7 Stereomicroscope System) and photographed using the Olympus SC50 digital camera for

total length. In both species, wholemount preparation was used to observe skin and yolk-sac surfaces, and sectional preparation was used to observe gill tissue.

a. Wholemount preparation

Dechorinated embryos were rinsed in 0.01M phosphate buffered saline (PBS) for 15min at room temperature, followed by another rinse in 30% sucrose/PBS solution, and a final rinse in PBS before a 30min incubation in 2% Normal Goat Serum.

b. Sectioning preparation

Similar to wholemount preparation, dechorinated embryos were rinsed in 0.01M PBS for 15min at room temperature, followed by another rinse in 30% sucrose/PBS solution before being placed into Cryomolds, covered with embedding medium, and snap frozen. Sections were cut at 5µm (-20°C) using Leica CM1860 UV Cryostat and placed on slides to dry. Slides were rinsed with PBS, following a 30min incubation in 2% Normal Goat Serum.

Antibody protocols were sourced from McCormick et al. (2003) and Hiroi et al. (2008). Slides and wholemounts were incubated in 1:1500 anti-Na, K-ATPase (alpha 5; deposited to the DSHB by Fambrough, D.M. (DSHB Hybridoma Product a5))/antibody buffer (0.01M PBS with 0.01% NaN_3, 0.1% BSA, 2% normal goat serum, and 0.02% keyhole limpet hemocyanin) overnight at 4°C. The following morning, the samples were rinsed with PBS and underwent a final incubation with 1:1000 Alexa 488 anti-mouse (Life Technologies Australia Pty Ltd, Mulgrave, VIC, AUS) for 2hrs at room temperature. Samples were rinsed with PBS and completed with a coverslip. For a full description of primary antibody collection, refer to McCormick et al. (2003).

Wholemounts and sectioned samples were examined on an inverted confocal fluorescent microscope (Zeiss LSM 710). Images were taken using Zen 2009 Light Edition (v 5.5.285) confocal software, using a combination of z-stacks and continuous imaging. To analyse images obtained from wholemount samples, ionocyte density and sizes were measured in ImageJ (v. 1.51s, National Institutes of Health, Rockville, MD, USA), and recorded in regard to their location. Specifically, the maximum area visualised for either the trunk or yolk-sac was measured and cells within the area were counted. Then, a calculation was performed to obtain an estimate per mm^2. Within the specified area, 12 ionocytes were randomly selected and measured. A grading system was used for sectional samples of gills

due to the complex structure of gills and the size of developing gill tissue. A grade was given of either, (0) for absent, (1) for sparse, (2) for moderately dense, or (3) for dense (Hiroi et al., 1998) to each sample after visually inspecting four individual sections.

Statistical Analyses

All statistical analyses were performed in R version 1.1.453. Linear mixed-effects models (LME) were used to determine changes in size and density estimates of Ionocytes throughout development. Models were determined by comparing all possible variations through the Akaike information criterion (AIC). Models were also visually assessed through Q-Q plots and residuals vs fitted plots to assess for normality and equal variances. In both species, density or size, and age were used as fixed effects, and species ID was included as a random effect for both yolk-sac and trunk data. A Tukey's multiple comparison test (glht, corrects *p*-values) was then used to find where significance differences occurred between ages.

4.3 Results

Extra-branchial ionocytes first appeared on the yolk-sac and trunk (i.e., the skin covering the main body compartment) at 2 dpf in *Ac. polyacanthus,* while ionocytes first appeared on the yolk-sac and trunk in *Am. melanopus* at 1 dpf (Figure 4-2; Figure 4-3). In *Ac. polyacanthus,* ionocyte densities on the trunk significantly increased with age, peaking at 4-5 dpf. Following 5 dpf, ionocyte densities on the trunk started decreasing (Figure 4-2; Appendix 6). The ionocyte densities on the yolk-sac remained similar across development, where significant differences were only found between 1 and 5 dpf, as well as between 1 and 9 dpf (Figure 4-2; Figure 4-3; Appendix 7). In *Am. melanopus,* no significant changes in ionocyte densities on either the trunk or yolk-sac were found (Figure 4-2; Figure 4-3; Appendix 9, 10); however, the ionocytes along both the trunk and yolk-sac significantly decreased in size over development (Figure 4-4; Appendix 11,12). Similarly, in *Ac. polyacanthus,* cell size on the yolk-sac decreased alongside increasing age; although, cell size at 9 dpf was not found to be significantly smaller than 5 and 6 dpf. Cell size along the trunk of *Ac. polyacanthus* was found to have some significant differences with progressive age; however, the trend was not the same as in other tissues. Cell size on the trunk of *Ac.*

polyacanthus was found to be significantly smaller at 5, 7, and 8 dpf when compared to 2 and 3 dpf, as well as cell size at 4 dpf being significantly smaller than cells at 3 dpf. Although, at 9 dpf cell sizes were not found to be smaller than cells at 2 and 3 dpf (Figure 4-4; Appendix 13, 14). In both species, regardless of cell size, ionocytes on the skin and yolk-sac appeared to form a multicellular complex, as suggested by the presence of multiple nuclei within an immune-reactive area (Figure 4-5).

In both species, branchial ionocytes were only found on the gill filaments (excluding pseudobranch) and not on the lamellae (Figure 4-6). ionocytes appeared as soon as filaments started to form, which occurred at 4-5 dpf in *Ac. polyacanthus* and 3 dpf in *Am. melanopus* (Figure 4-7). Moderately to highly dense clusters of ionocytes were found on the filaments at 7 dpf in *Ac. polyacanthus* and 5 dpf in *Am. melanopus*, simultaneous to the formation of lamellae (Figure 4-7).
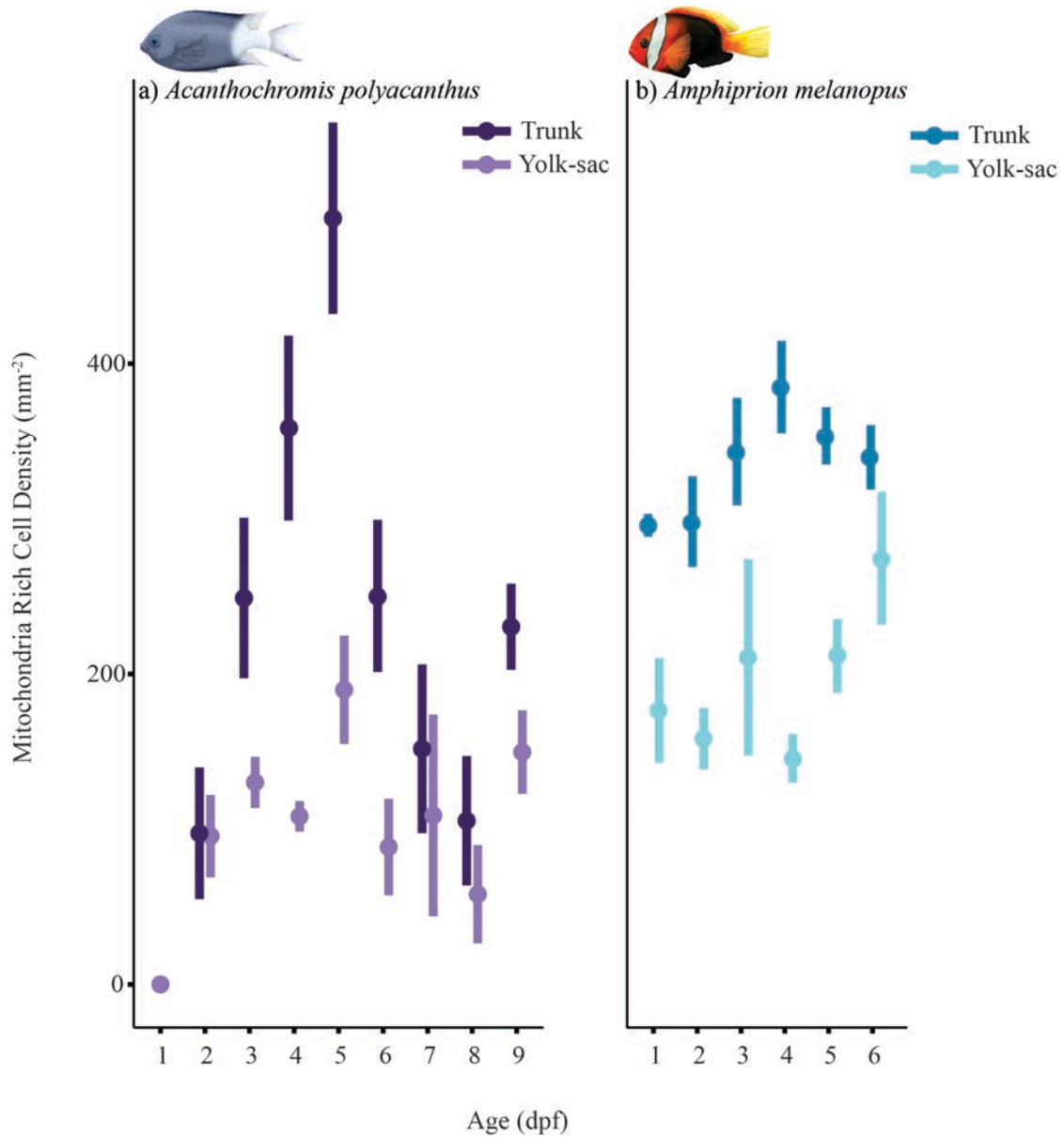
Figure 4-2: Changes in ionocytes (mitochondria rich cell) densities on the yolk-sac and trunk in: **a)** *Acanthochromis polyacanthus* and **b)** *Amphiprion melanopus*. Points represent means with ± SE.
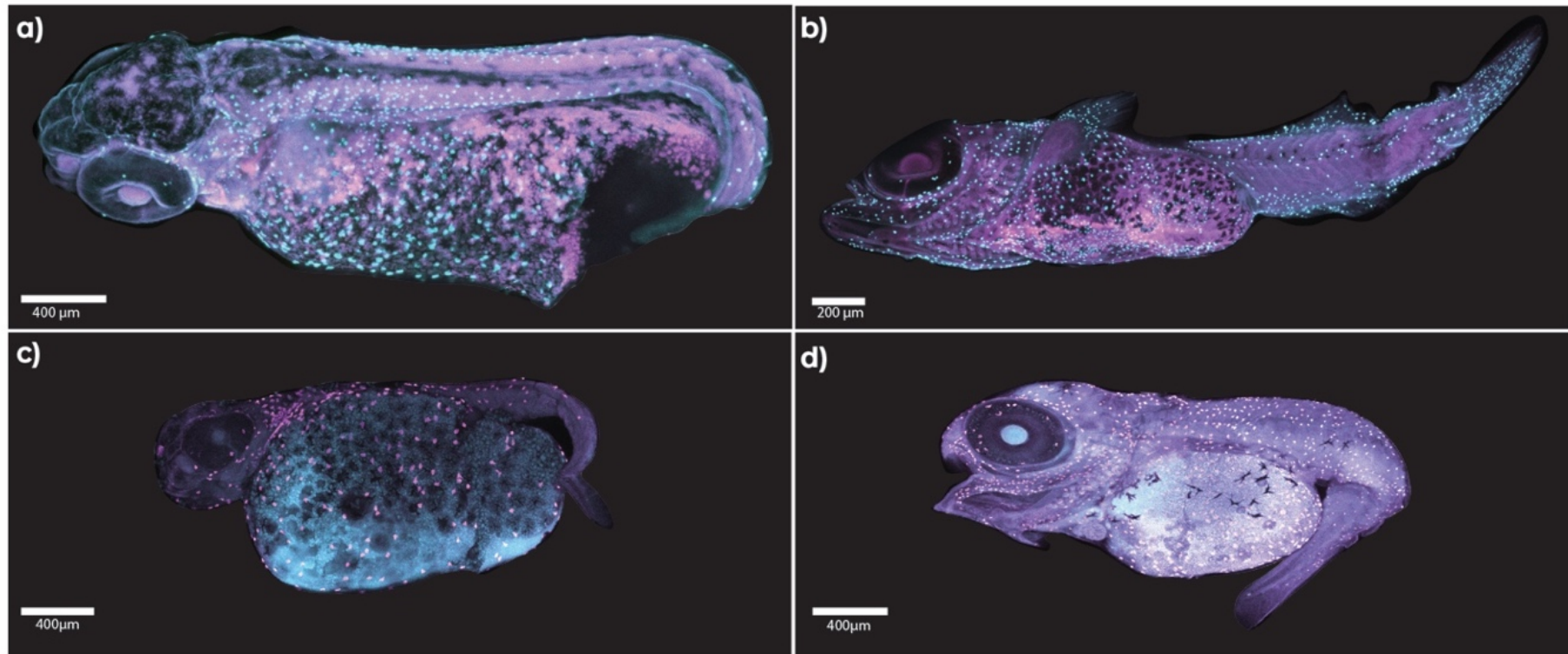
Figure 4-3: Na, K-ATPase (blue) immunoreactivity along the external structures (skin and yolk-sac) in *Ac. polyacanthus* embryos at **a)** 5dpf and **b)** 7dpf. Na, K-ATPase (pink) and immunoreactivity along the external structures (skin and yolk-sac) in *Am. melanopus* embryos at **c)** 3dpf and **d)** 6dpf.
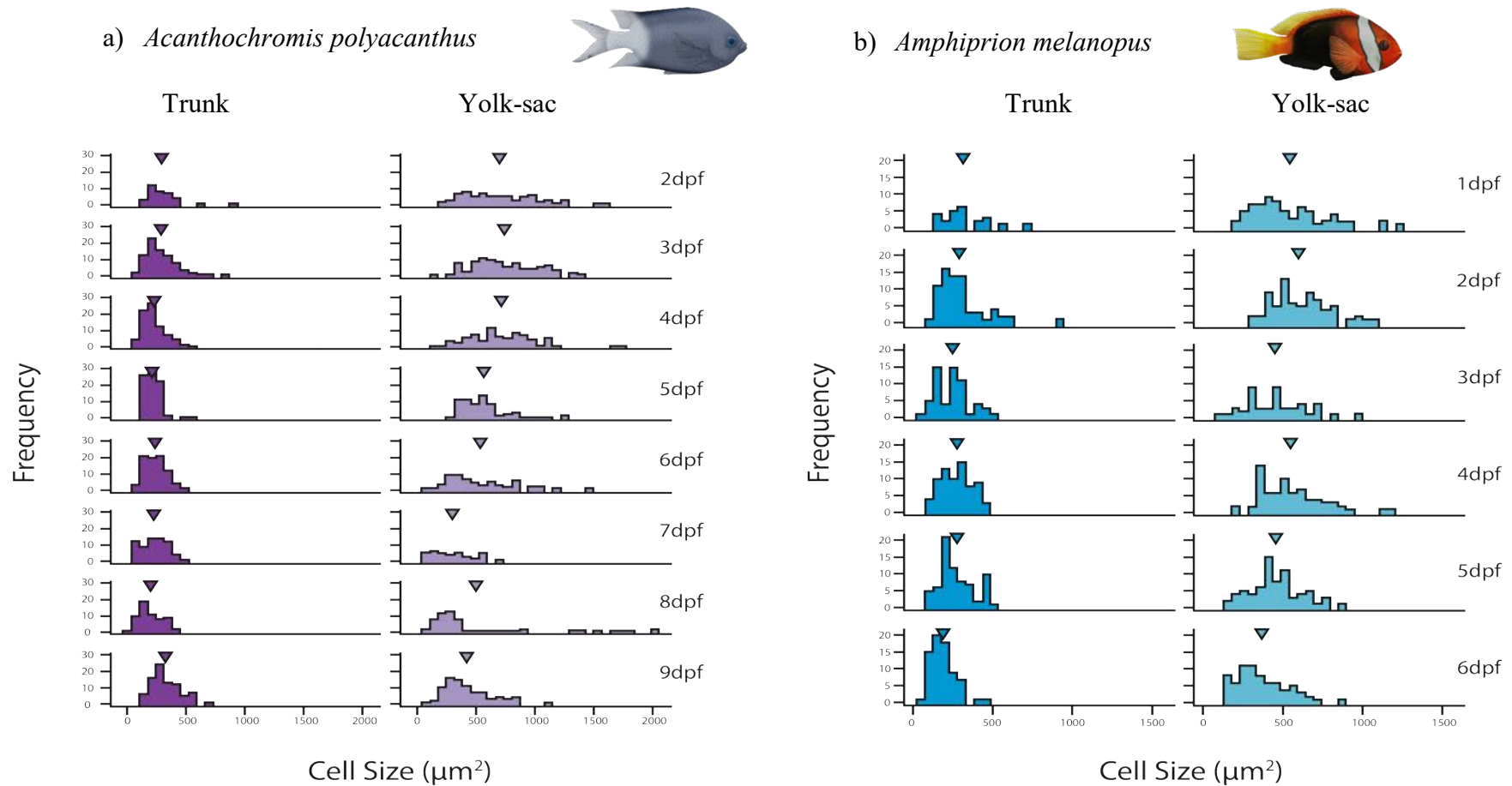
Figure 4-4: Frequency histogram showing changes in ionocytes (mitochondria rich cell) size through age on the yolk-sac and trunk in: **a)** *Acanthochromis polyacanthus,* and **b)** *Amphiprion melanopus.* Arrows indicate mean cell size of mitochondria rich cells in regards to the corresponding location.
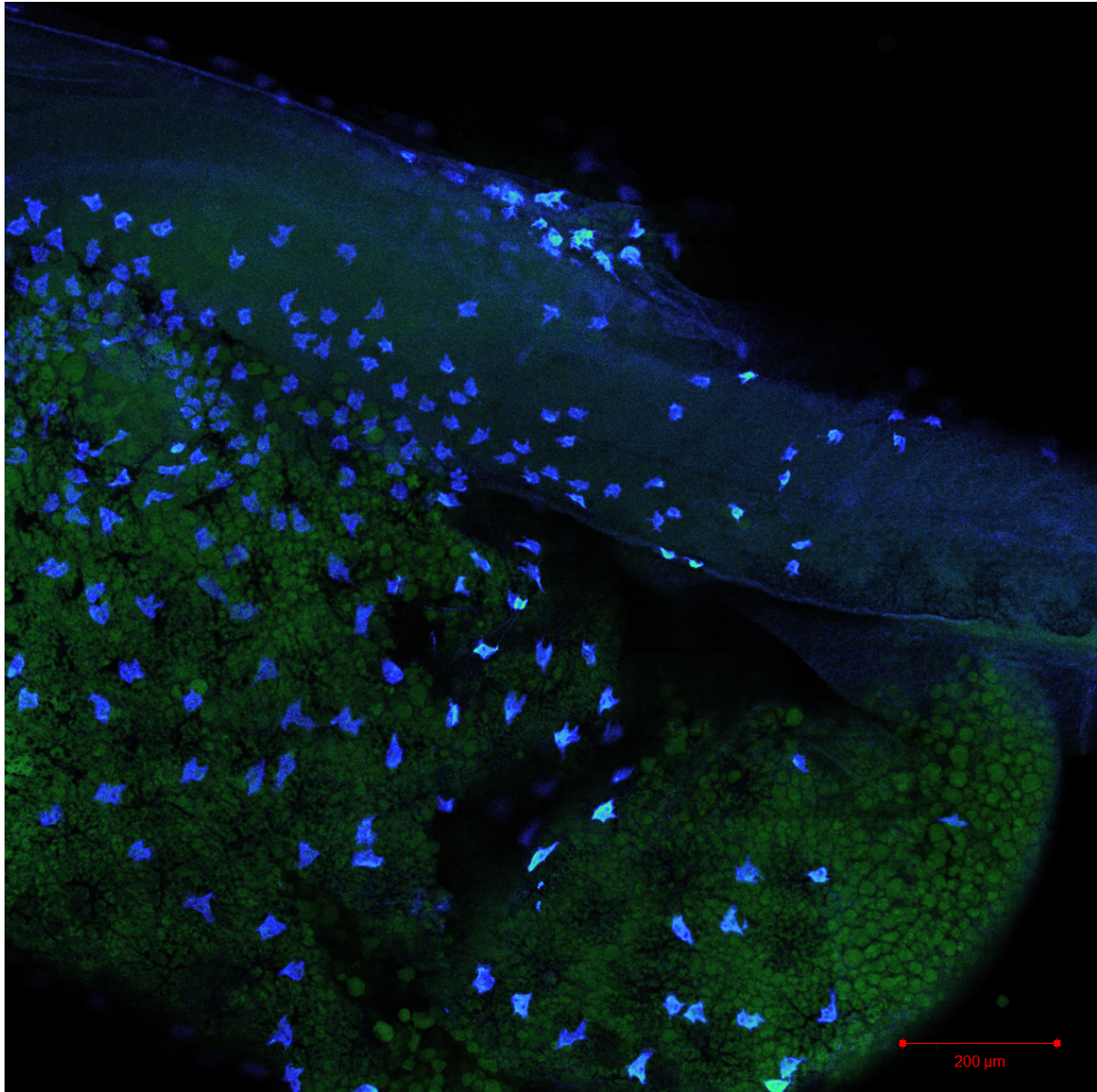
Figure 4-5: Immunoreactive (blue) area on the trunk and yolk-sac of 2dpf *Amphiprion melanopus* embryo. Micrograph shows ionocytes (mitochondria rich cells) to form multicellular complexes, as depicted by more than one immunonegative nuclei within the immunopositive mitochondria rich cells.
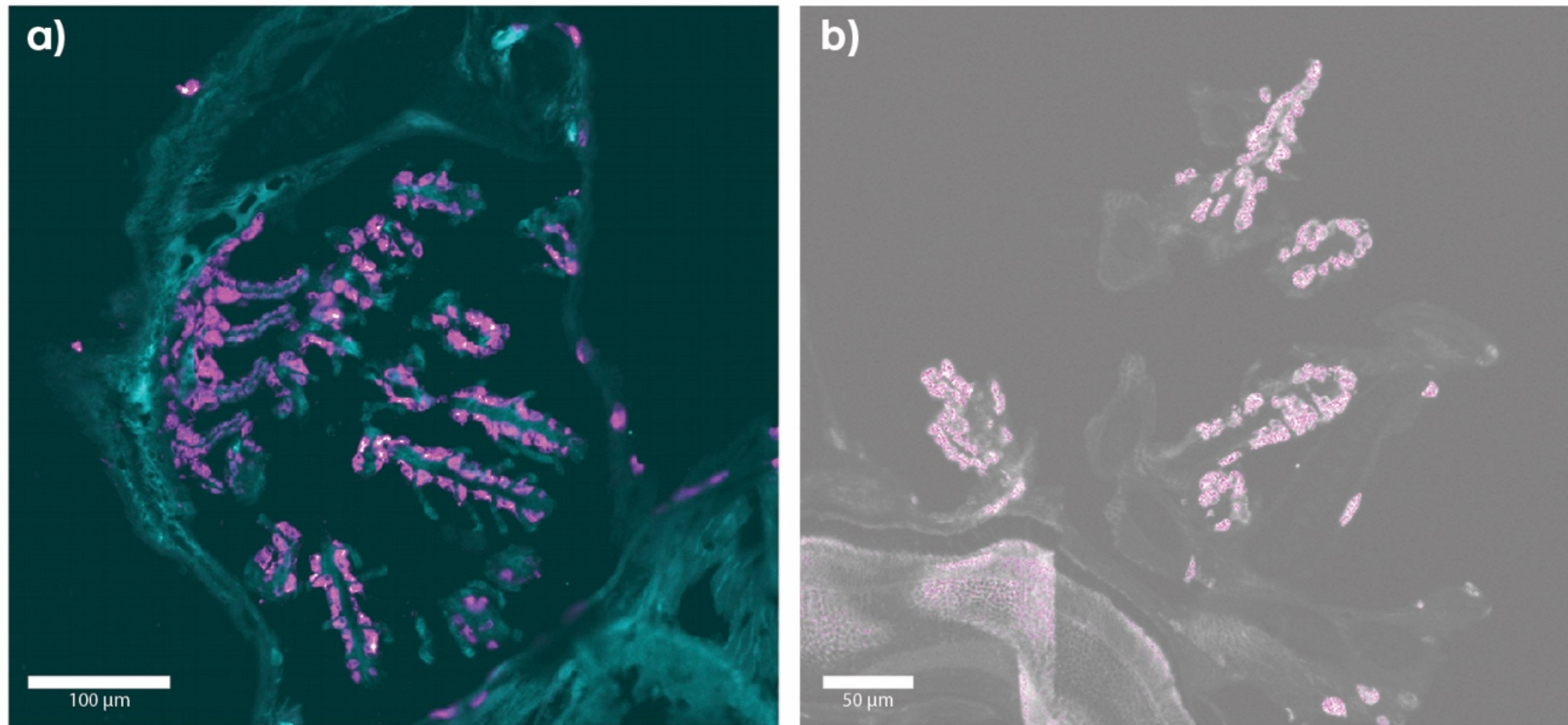
Figure 4-6: Na, K-ATPase (pink) immunoreactivity in the gills of **a)** *Ac. polyacanthus* embryos 7dpf, and **b)** in *Am. melanopus* embryos 6dpf. Enzyme activity was only found along the filament tissue of the lamellae, excluding pseudobranchial tissue.
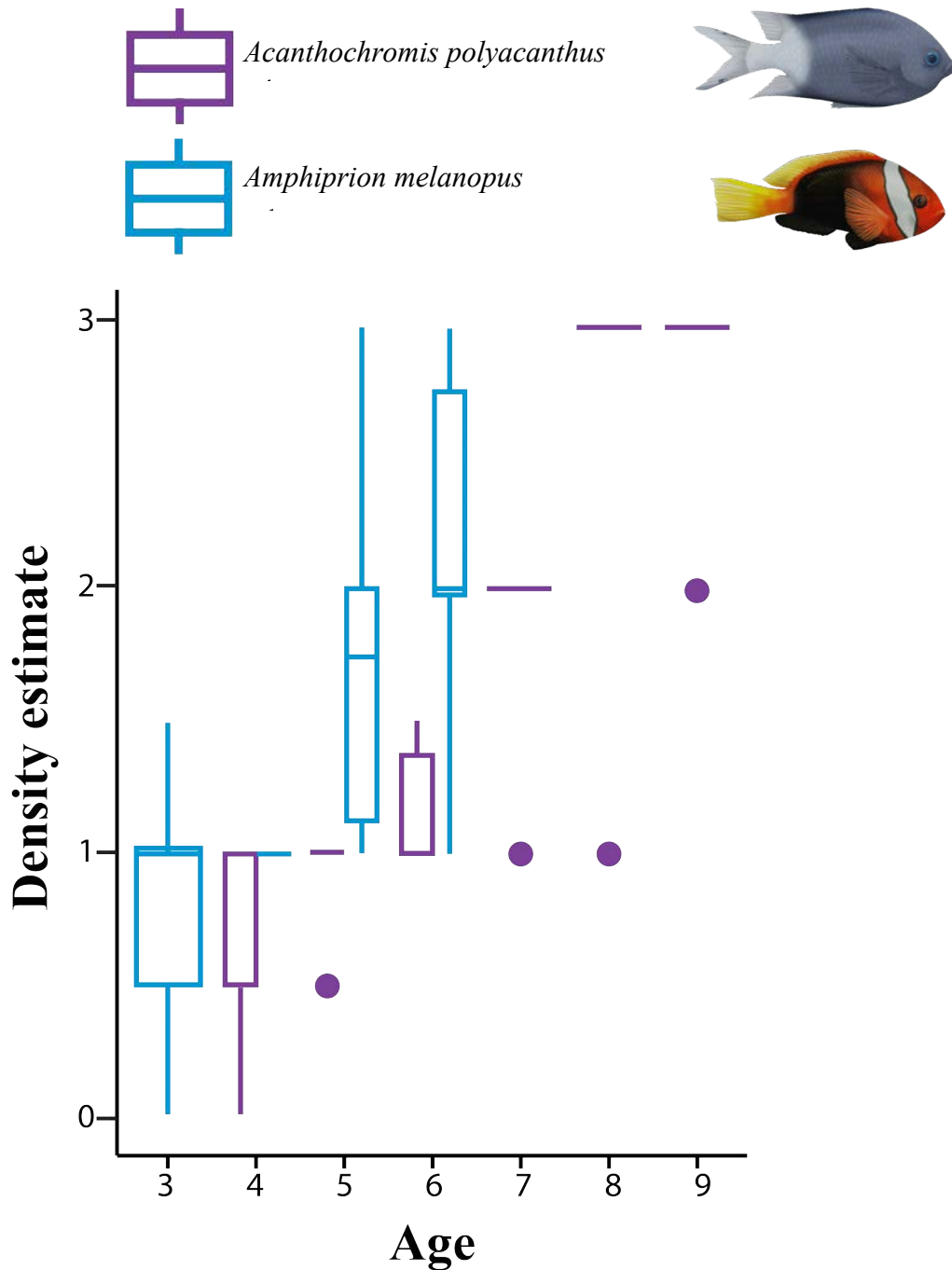
Figure 4-7: Changes in ionocytes (mitochondria rich cell) densities on the gill filaments in: *Acanthochromis polyacanthus* (purple) and *Amphiprion melanopus* (blue). Boxplots represent median, upper and lower quartiles, and outliers. Density estimates: (0) absent, (1) sparse, (2) moderately dense, (3) dense.

4.4 Discussion

Coral reef fishes develop extra-branchial ionocytes during the early stages of embryonic development. Ionocytes were found on the trunk and yolk-sac at 2 dpf in *Ac. polyacanthus* embryos and 1 dpf in *Am. melanopus* embryos. This is the first evidence that coral reef fishes obtain extra-branchial ionocytes for ion regulation, although similar observations have been made in other species (Hiroi and McCormick, 2012, Hiroi et al., 2005). Like in other species, the ionocytes along the yolk-sac and trunk were found to form multicellular complexes (Figure 4-5), which include the main chloride cell and adjacent accessory cells (Shiraishi et al., 1997, Hiroi et al., 1998, Kaneko et al., 2002) . The formation of multicellular complexes in extra-branchial ionocytes is consistent with branchial ionocytes, which produce 'leaky tight junctions' between the ionocyte and accessory cells to aid Na^+ excretion (Figure 4-1). The chemical and morphological evidence presented here, therefore, suggests that extra-branchial ionocytes are likely to perform ionoregulatory functions before these tasks transition to the gills.

Ion regulation in fishes is required to transition from cutaneous to branchial pathways with growth due to geometric constraints. Diffusion across the skin and yolk-sac epithelium becomes limiting, as the organism gets larger due to declines in SA/V and increasing distance between the exterior and blood where ionocytes are unable to reach both surfaces and thus perform appropriately (Hiroi et al., 1998, Alderdice, 1988). At this point, ionocytes and thus ionoregulatory processes transition to the gills, as they evade these constraints. In the two study species here, ionocytes were found to form on the gill filaments simultaneously to filament formation and were not found on the lamellae, an observation that is common in most marine fish species (Uchida et al., 1996, Hwang and Lee, 2007, Perry, 1997, McCormick et al., 2003). This timing, approximately at 3-4 dpf, could be depicting when ionoregulatory processes start to become limited through cutaneous processes, and therefore, migrate to the gills. Morphological trends in ionocytes such as, declining density and cell size, have been found to indicate the transition and commencement of branchial ion regulation (Rombough, 2007, Hiroi et al., 1998). Changes in both density and cell sizes were found in both species investigated here, where declines in density and cell size occurred in ionocytes located on cutaneous pathways (i.e., skin and yolk-sac) concomitantly with

substantial increases in ionocyte densities on branchial pathways (i.e., gill filaments). Together, these trends depict the onset of ion regulation transitioning from cutaneous to branchial pathways and appears to occur before hatching in both species.

In these two coral reef fish species, ionoregulatory processes at both cutaneous and branchial locations develop faster than in any other studied fish thus far. Extra-branchial ionocytes develop as early as 1 dpf in *Am. melanopus* and 2 dpf in *Ac. polyacanthus*, where ionocyte development in *Am. melanopus* beats the previous record of 2 dpf in tipalia (Hwang et al., 1994, Ayson et al., 1994). Similarly, for branchial ionocyte development, I found ionocytes to appear on the gills as early as 3 dpf in both species, beating the previous record of 5.5 dpf in zebrafish (Rombough, 2002). It is uncertain as to why these species exhibit such fast-paced developmental patterns for ion regulation, as freshwater species are often found to develop branchial ionoregulatory processes before marine fishes. This is thought to occur because the ability to obtain ions in ion-deficient environments (i.e., freshwater) is more difficult than the ability to remove ions into a high ion-associated environment (i.e., seawater). However, it is important to consider other gill-mediated processes such as acid/base regulation and ammonia excretion, given they are often coupled with ionoregulatory processes (Randall, 2005). These processes are yet to be investigated not only in coral reef fishes, but in teleost fishes in general. Although, movements of both Na^+ and NH_4^+ in developing rainbow trout were found to transition to the gills from cutaneous pathways simultaneously at 15 dph, and both transitioning before O_2 uptake (Zimmer et al., 2014). This suggests that the removal of nitrogenous wastes is also an important role during early gill development.

In regard to coral reef fishes, these processes (i.e., acid/base regulation and ammonia excretion) are likely to be extremely important early in development, and therefore, may play influential roles in gill development. Indeed, coral reef fishes are likely to produce high loads of metabolic wastes during embryonic life stages because of the associated high growth rates and yolk-sac consumption during embryonic development and the commencement of exogeneous feeding upon hatching (Donelson et al., 2012, Green and McCormick, 2001). These processes produce lethal nitrogenous wastes that must be removed efficiently (Zimmer and Wood, 2015, Randall and Tsui, 2002). Additionally, the onset of extensive aerobic activities that occurs upon hatching would also contribute, as acidic waste is generated from

cellular respiration (Clanton et al., 2013). Consequently, my study shows branchial ion regulation occurs early in development; however, identifying the exact cause driving this early development requires further investigation.

Conclusion

Overall, this study shows that the developmental sequence of ion regulation in coral reef fishes occurs faster than any other teleost fish investigated. Moreover, this study provides critical support that ion regulation may be the foremost process to function at the gills in developing coral reef fishes. Although, additional investigation is required to tease apart the underlining reasons for ionoregulatory mechanisms to function at the gills early and before O_2 uptake (chapters 2 & 3). This information will improve our understanding of gill development during early ontogeny, which processes become limited early in development, and perhaps, the most vulnerable developmental stages.

5 General Discussion

Oxygen uptake and ionoregulatory processes are two of the most important physiological systems in nearly every organism, given they underpin mechanisms for fitness and survival. In fishes, both of these processes are primarily performed at the gills, at least in adult stages (Evans, 1998, Evans et al., 2005); however, understanding when and in what sequence these processes transition to the gills is relatively unexplored. This thesis was designed to investigate structural and functional aspects of gills in developing coral reef fishes to gain insight into which process drives gill formation and thus becomes limited first. The results obtained throughout this thesis provides, for the first time, insight into the development of two key physiological processes – oxygen uptake and ion regulation – in developing coral reef fishes. The results outline a critical developmental window in coral reef fishes and suggests how this developmental window may contribute to vulnerability experienced during these early life stages.

Chapter two investigated the structural development of gills in two coral reef fishes during early life stages. This chapter was crucial for the entire thesis, as it provided the backbone to the following two chapters. Describing the sequence and timing of each gill structure determined the beginning, ending, and overall duration of gill development in the two study species and was able to be used as a reference in later chapters, three and four. In chapter three, I measured oxygen uptake rates in developing embryos with and without functional Hb to determine when gills are required for oxygen uptake. Knowing when gills begin functioning for oxygen uptake, I was then able to compare against other gill-mediated processes, such as ion regulation – measured in chapter four – to determine which function becomes limited first and thus, requires gills earlier. In chapter four, I identified when ion-regulating cells appeared on cutaneous and branchial pathways through immunohistochemistry. Additionally, I used morphometric assays on cells to detect trends in cell abundance and size that occurs with development. Together, the results from all three data chapters are discussed below and presented in a timeline (Figure 5-1; Figure 5-2).

The development of gill structures (obtained in **Chapter two**) in *Ac. polyacanthus* and *Am. melanopus* was complete within the embryonic phase, and to my knowledge, occurred faster than any other studied fish species. Gill development was structurally complete at 5 dpf and 7 dpf in *Am. melanopus* and *Ac. polyacanthus*, respectively, i.e., 1-2 days prior to hatching. Tilapia was previously titled the fastest gill developer with the first signs of gills noted at 7 dpf (2 dph) (Li et al., 1995). Additionally, the transition from cutaneous to branchial pathways for both oxygen uptake (**Chapter three**) and ionoregulatory processes (**Chapter four)** also occurred at a rapid pace. I found ionocytes for ionoregulatory processes to transition to the gills simultaneously to the formation of gill arches and filaments at 3 dpf, also beating the zebrafish (5.5 dpf; (Rombough, 2002). This transition occurred during embryonic development, and therefore, it would be impossible to obtain direct measurements of ion movements between cutaneous and branchial pathways. However, morphological trends of ionocytes can be used as a tool to determine the onset of ionoregulatory processes at the gills (Brauner and Rombough, 2012) when direct measurements are unable to be attained. Furthermore, gills begin primarily performing O_2 uptake roles upon hatching. This also appears to be the earliest timing for any fish species to rely on gills for oxygen uptake. But more interestingly, ionocytes forming on the gills – perhaps indicating the onset of branchial ion regulation – occurred before O_2 uptake. Similar to other previously studied fishes (Reviewed by Rombough (2007), it suggests, for the first time, that the trend exhibited by coral reef fishes supports the ionoregulatory hypothesis; however, gill development overall occurs faster than any other studied fish species to date.
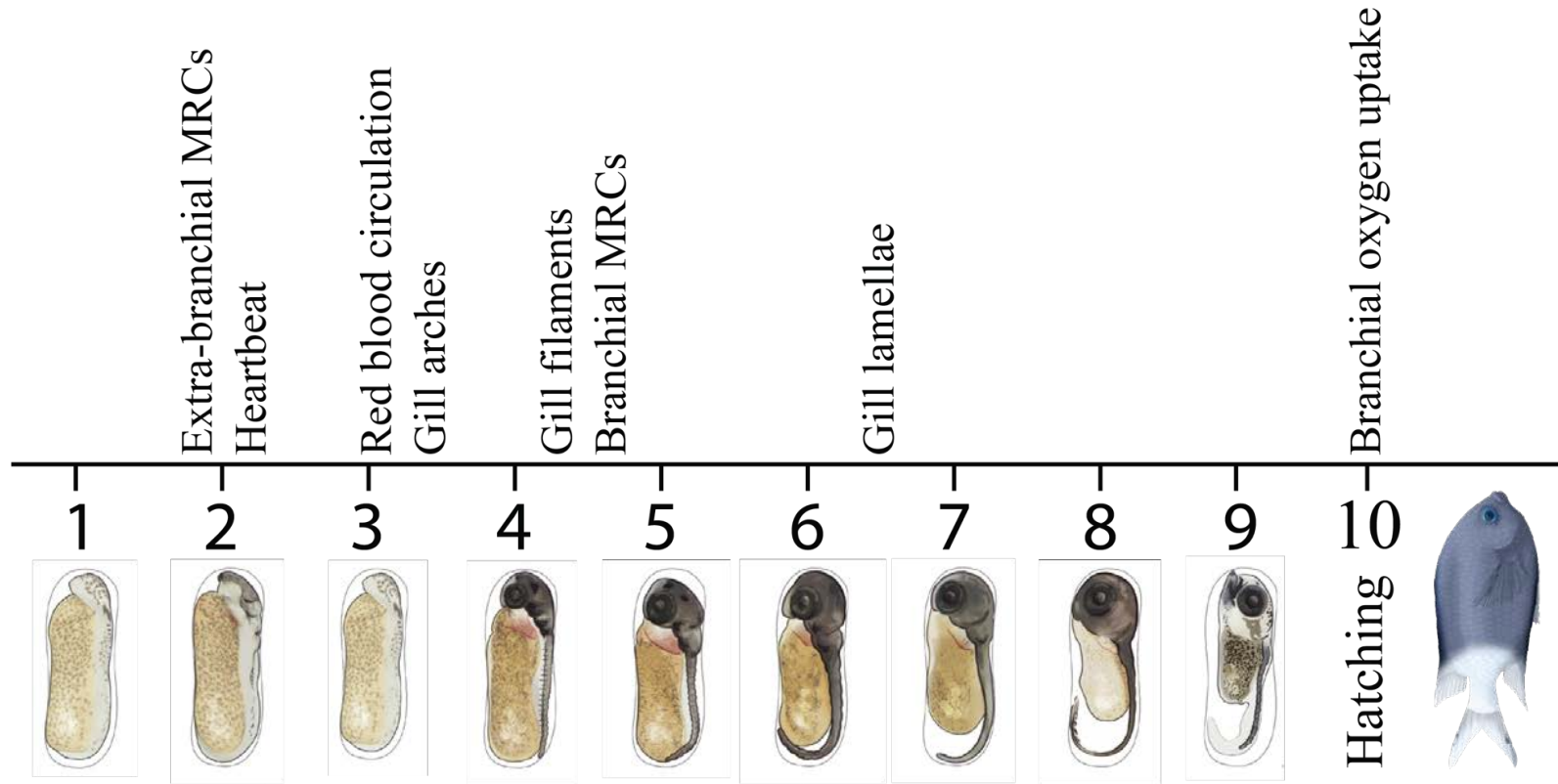
Figure 5-1: Developmental timeline of *Acanthochromis polyacanthus*. Ionocytes: mitochondria-rich cells (MRCs).
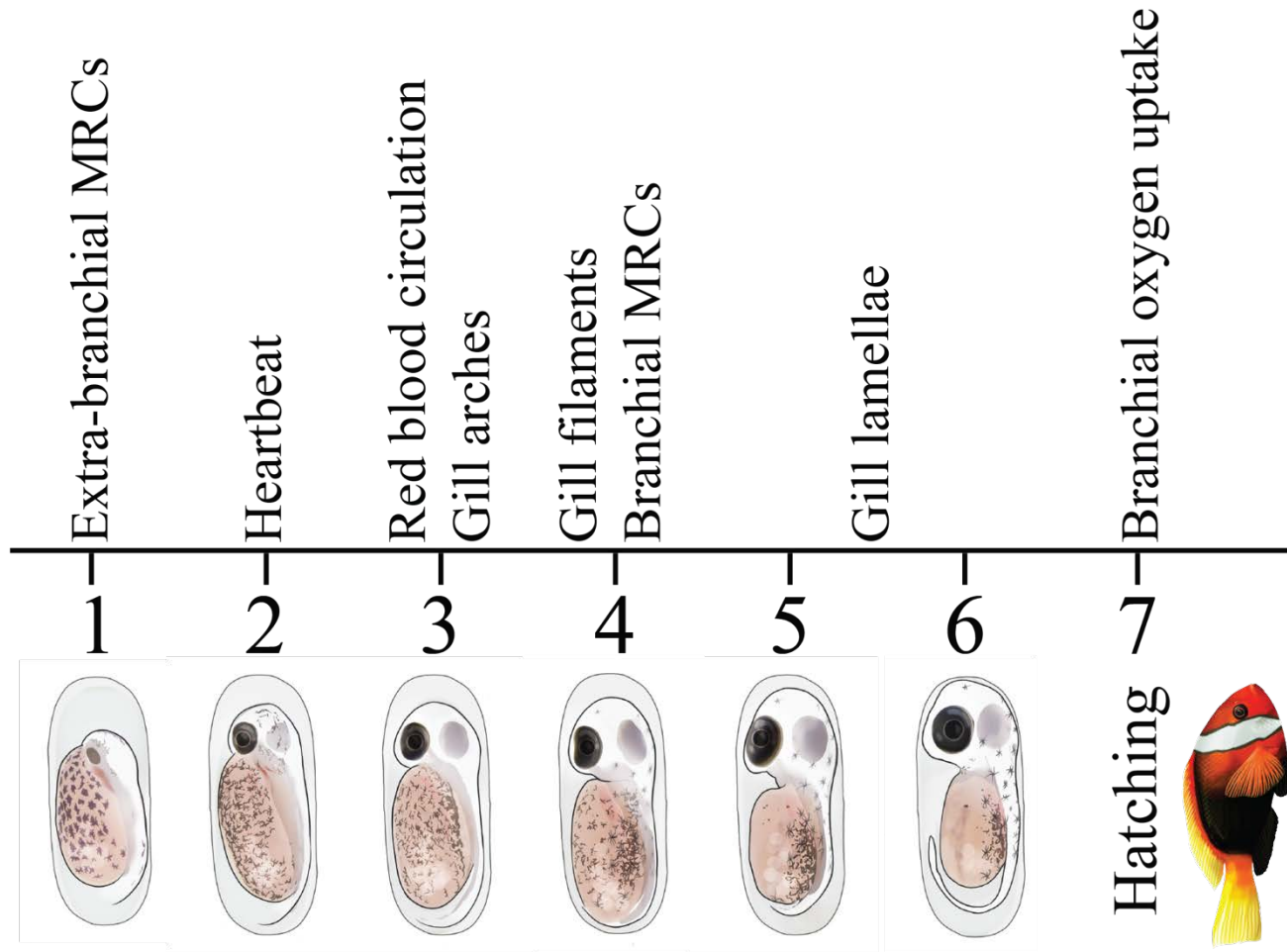
Figure 5-2: Developmental timeline of *Amphiprion melanopus*. Ionocytes: mitochondria-rich cells (MRCs).

It would be expected to relate these accelerated development patterns to environmental factors such as temperature and salinity, as coral reef fishes and the previous record holders – zebrafish and tilapia – reside in contrasting environments. However, temperature regimes between both zebrafish and tilapia and the two current species are comparable, i.e., reared at 28°C, removing temperature as a potential factor contributing to this trend. Additionally, salinity levels are unlikely to be the driver behind early gill formation in the two coral reef fish species, as previous studies find freshwater fishes to develop branchial ionocytes before marine fishes (Rombough, 2007). This is thought to occur because the ability to obtain ions in ion-deficient environments (i.e., freshwater) is more difficult than the ability to remove ions into a high ion-associated environment (i.e., seawater). Although, when tilapia embryos were raised in different salinities (i.e., freshwater vs. seawater), extra-branchial ionocytes appeared simultaneously at 2 dpf, but more interestingly, the density and size of ionocytes were larger in seawater-raised embryos than their freshwater counterparts; however, decreases in density and size occurred earlier in freshwater-raised tilapia, perhaps suggesting ionoregulatory mechanisms are becoming limited earlier and therefore are required to transition to the gills before marine raised tilapia (Ayson et al., 1994). This is thought to occur because during embryonic development, the yolk-sac supplies sufficient ion levels; however, upon hatching and yolk-sac absorption, ion levels in fishes decrease substantially, where then, they have to rely on their environment to maintain these levels. However, for freshwater fishes, their environment is ion deficient, thus necessitating reliable and more efficient methods for obtaining ions earlier than when marine fishes would require gills for ion removal (Rombough, 2007). Therefore, my data alludes that other aspects beyond geometric constraints (i.e., decreasing surface area to volume ratio) may demand efficient ionoregulatory processes and consequently, the early onset of branchial ion regulation.

Another aspect to consider – that was not measured in this thesis – is other ion regulating processes such as acid/base regulation and ammonia excretion, given they are often coupled with ion/osmoregulatory processes (Randall, 2005). These processes are yet to be investigated, not only in coral reef fishes, but also relatively unexplored in teleost fishes. In a recent study by Zimmer and colleagues, movements of both Na^+ and NH_4^+ in developing rainbow trout larvae were found to transition from cutaneous pathways to the gills simultaneously at 15 dph, but before O_2 uptake, which occurred at 27 dph (Zimmer et al.,

2014). This suggests that removing nitrogenous wastes is also an important and demanding process during development. Indeed, high loads of metabolic wastes, in particular lethal nitrogenous wastes, accumulate during these early developmental stages resulting from cellular processes associated with high growth rates (e.g., organo- and morphogenesis) and yolk-sac consumption (Wright et al., 1995, Dhiyebi et al., 2013, Zimmer, 2014). Both processes occur rapidly in coral reef fishes (Green and McCormick, 2001, Donelson et al., 2012). Therefore, developing coral reef fishes would require established methods to remove these wastes before they reach toxic levels (Randall and Tsui, 2002). High growth rates and yolk-sac consumption are most notably driving early gill development for ionoregulatory requirements, but branchial respiration was found to also occur exceptionally early in development in these two coral reef fish species (chapter three).

The underlying reasons supporting ionregulatory mechanisms to transition to the gills are not directly linked to the early onset of respiratory advancements, where a more suitable reason driving early branchial oxygen uptake may be related to the unique early life history traits these fishes possess. Larval coral reef fishes, upon hatching, undergo major advancements in aerobic capacity and activities that are likely to be underpinning early gill development, both structurally and physiologically. These gill-demanding activities – commonly found in pomacentrids – include enhanced $\dot{M}O_2$ (e.g., 5250 mg kg^{-1} h^{-1}) that are currently the highest $\dot{M}O_2$ recorded in ectothermic vertebrates, the fastest sustained swimming speeds (50 BL sec^{-1}), and upon settlement (i.e., transition from pelagic to reef association), are among the most hypoxia tolerant species investigated to date (Stobutzki and Bellwood, 1994, Nilsson et al., 2007, Nilsson, 2010). Specifically, these traits are found in species that undergo a pelagic larval duration (PLD), where they spend weeks to months in the open ocean before finding suitable habitat on coral reefs. My study species, *Am. melanopus* exhibits this life history trait. Therefore, we can assume that *Am. melanopus* also possess these traits post-hatching. In contrast, *Ac. polyacanthus* does not exhibit this life history trait and rather, upon hatching, immediately begins life on the reef (Leis and McCormick, 2002). Despite *Ac. polyacanthus* lacking a PLD, upon hatch, they still exhibit these gill-demanding activities. For instance, *Ac. polyacanthus* exhibits profound swimming abilities (unpublished data, Downie et al) and enhanced oxygen uptake rates (e.g., 2553 mg kg^{-1} h^{-1}) that are equivalent to large pelagic fishes, such as tuna (Nilsson et al., 2007). More interestingly, hypoxia tolerance traits advance when larval coral reef fishes transition from

the pelagic to the reef habitat (i.e., settlement) and progress throughout adulthood to withstand nightly periods of low oxygen. Although, *Ac. polyacanthus* resides directly on the reef after hatching, they still gain these hypoxia tolerance traits that would require functional gills immediately (Nilsson and Ostlund-Nilsson, 2004, Sollid and Nilsson, 2006). Withstanding hypoxia requires a high Hb affinity and a large respiratory surface area to sufficiently obtain oxygen in oxygen deficient environments (Randall et al., 2014, Sollid and Nilsson, 2006). This supports my results of oxygen uptake transitioning to the gills upon hatch. Overall, rapid gill development found in the two study species is likely occurring, first, to sustain high growth rates and remove excessive bodily wastes during embryonic development, then second, to meet the demands of high-performance and withstanding challenging environmental conditions that coral reef fishes endure post hatch.

The onset of gill structures occurs in sequence of ion regulatory tissue (i.e., filaments) before respiratory tissue (i.e., lamellae) and mirrored by the transition of their accompanied functions; however, before this timing, the heart begins to beat, and blood circulation commences. The role of these features is unknown during this time, but is in support of the prosynchronotropy hypothesis. The prosynchronotropy hypothesis states that the early onset of a beating heart and red blood circulation occurs well before it is required for the transport of gases, nutrients, and wastes, perhaps to enhance cardiac growth and/or angiogenesis (i.e., development of new vessels from present ones; (Burggren, 2004). Results obtained here support this hypothesis, where a beating heart and red blood circulation commenced extremely early in the embryonic phase at 2-3 dpf in both species, before they were needed for transportation. So, if the role of a beating heart and red blood circulation is to enhance cardiac growth and/or angiogenesis, before it is needed in transport, why are Hb molecules present?

Considering that the main role of Hb is to enhance O_2 delivery, I would expect that if Hb is being used, there would be a significant difference in $\dot{M}O_2$ between control and PHZ treated (i.e., Hb inhibited) embryos. My results found $\dot{M}O_2$ to remain similar between control and PHZ treated embryos (i.e., periods of cutaneous respiration), indicating that at this stage Hb is not playing a significant role in O_2 uptake, in either cutaneous or branchial pathways. Perhaps, Hb is not utilize for cutaneous respiration because cellular conditions within this

area of the body (e.g., low pH) would favour O_2 release from Hb (via the combined Bohr and Root effects) rather than O_2 loading (Randall et al., 2014, Brauner and Randall, 1996, Nikinmaa, 2011). These changes, prompting the Bohr and Root effects, are predominantly initiated by increases of waste CO_2 – a product of aerobic metabolism (Nikinmaa, 2011, Hilpert et al., 1963, Root, 1931). In developing coral reef fishes, it may be predicted that high loads of metabolic waste may be present within areas associated with cutaneous respiration because of high growth rates and yolk-sac consumption (Donelson et al., 2012, Green and McCormick, 2001). As a result, lowered pH and other cellular conditions may favour oxygen offloading parallel to later life stages. Therefore, Hb use would be counter-productive.

The presence of Hb appearing early in development has been seen in the embryonic stages of several fish, amphibian, and bird species, where Hb appears before it is used to enhance O_2 uptake (reviewed by Burggren 2004). In fishes, it is suggested that the early presence of Hb may be involved in the initial inflation of the swim bladder (Pelster and Burggren, 1996). The mechanism behind inflating the swim bladder for the first time can either occur by fishes gulping air at the water surface (physostomous species) or using specialised structures such as gas glands and the *rete mirabile* (physoclistous species) to inflate their swim bladder. While it has not been extensively studied among fish species, it appears that many freshwater fishes use an air gulping method, while marine fishes mainly use specialised structures to first inflate their swim bladder (Woolley and Qin, 2010). In the two study species here, it is presumed although not known that they are most likely to be physoclistous species. In particular, *Ac. polyacanthus* larvae reside by their mother's side for protection upon hatching; therefore, it is unlikely that the larvae would leave their mother's protection and swim to the surface to inflate their swim bladder through air gulping. If the two study species here were to use Hb early in ontogeny to initially inflate their swim bladder, it would be predicted that lower oxygen uptake rates would occur in embryos with inhibited Hb compared to control embryos. My results found oxygen uptake rates to remain similar across embryos with and without Hb. However, the amount of oxygen required to inflate the swim bladder during early development may be insignificant to the total amount of oxygen required for a developing fish and therefore is not detected. Unfortunately, swim bladder inflation was not measured in this study, but does require further investigation to determine whether Hb plays an immediate role in inflating swim bladder before primarily enhancing branchial respiration.

Another possible reason for Hb to develop so early in embryonic coral reef fishes may be to ensure sufficient oxygen is obtained during nightly periods of low oxygen conditions. As mentioned earlier, coral reef fishes can tolerate comparatively low oxygen levels, as their Hb exhibits a high affinity for oxygen and a low P_{50} (oxygen tension at which Hb is 50% saturated; Nilsson and Ostlund-Nilsson, 2004, Sollid and Nilsson, 2006). This trait may be apparent during the embryonic stages of coral reef fishes to maintain oxygen uptake, as they are benthic egg layers (Leis and McCormick, 2002); thus, their ability to move and seek more well-oxygenated habitats is restricted. Although, further investigation is required to determine whether this trait exists during coral reef fish early life stages. If this trait is apparent in embryonic coral reef fishes, it may be argued, why is branchial respiration not utilized throughout development. Therefore, it is important to discuss whether it is beneficial for a developing organism to have functional gills early, as the cost to benefit ratio may be unfavourable. The cost of overcoming physical barriers, such as the chorion and boundary layers, as well as other environmental parameters may outweigh the benefits of utilizing gills during this life stage. Although, some evidence shows that movements (e.g., O_2) between the chorion and boundary layers are sufficient for a developing embryo. For example, minimal differences (e.g., <5%) were found between O_2 tensions within the free-flowing water and an embryo's boundary layer (Rombough, 1989, Ciuhandu et al., 2007), and as O_2 content decreases within the boundary layer (i.e., due to embryonic respiration), it is replenished instantaneously by O_2 present in the surrounding water (Daykin, 1965, Pinder and Feder, 1990). This suggest that the permeability of an embyro's chorion does not limit their development. Nonetheless, other environmental properties are more likely to be costly. For instance, fish during early development are small aquatic organisms with high SA/V, which creates immense drag, therefore, adopting a low Reynolds number (ratio of inertial forces to viscous forces) (Vogel, 1994). This principle directly applies to the ability of the organism to flush water across its gills, where a small organism with a large surface area, including the gill surface area, would require great force to move a body of viscous fluid (i.e., water) across the gills. Therefore, during these early developmental stages, it may be unfavourable and energetically costly to utilize gills, in particular for oxygen uptake and to some extent, for ion regulation.

Overall, my research found that ionoregulatory processes appear to be the primary function of gills in developing coral reef fishes, perhaps related to high growth rates and yolk-sac consumption; whereas, larval life traits are the primary driver behind the early onset of branchial oxygen uptake. Based on the prevalence of unique larval abilities in coral reef fishes, I suspect these fast-paced developmental patterns may extend beyond the two study species and pomacentrids. Indeed, nearly all coral reef fishes undergo a pelagic larval duration, where increased oxygen uptake rates and profound swimming abilities are most likely to occur (Leis and McCormick, 2002, Stobutzki and Bellwood, 1997, Nilsson et al., 2007). In particular, many coral reef fishes have been found to possess these strong swimming abilities, which could be linked to a proficient respiratory system. Swimming abilities are influenced by the proportion of red muscle, extent of circulatory system, and possessing a relatively large respiratory surface area (Wood and McDonald, 1997). Therefore, rapid gill development may be common across all coral reef fishes in order to support these early life traits.

Furthermore, the results obtained throughout this thesis have started to fill several knowledge gaps within the field of developmental fish biology. This thesis focused on a substantial fish group (i.e., coral reef fishes) that lacks understanding in fundamental mechanisms during ontogeny but are facing some of the most intense anthropogenic stressors. Therefore, this study emphasises how physiology can be used in conservation-based studies and ultimately, improve predictions for future coral reef ecosystems. For example, this thesis addressed when critical physiological processes develop in coral reef fishes and outlined a critical developmental window, which can be targeted in future investigations. Future studies, therefore, will be obtaining results that are relevant for predicting future fish populations, given that the physiological processes focused on throughout this thesis underpin mechanisms for fish fitness and survival. Furthermore, investigating how perhaps, the most vulnerable life stage will develop and respond in the face of intensifying anthropogenic stressors can help build toward more accurate and reliable understanding of coral reef ecosystems and generate more reasons to mitigate anthropogenic stressors.

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7 Appendices

Appendix 1 Micrographs of *Ac. polyacanthus* embryos at 3 dpf showing: a) control embryo with red blood (haemoglobin present) and b) phenylhydrazine exposed embryo with yellow-clear blood (haemoglobin destroyed).
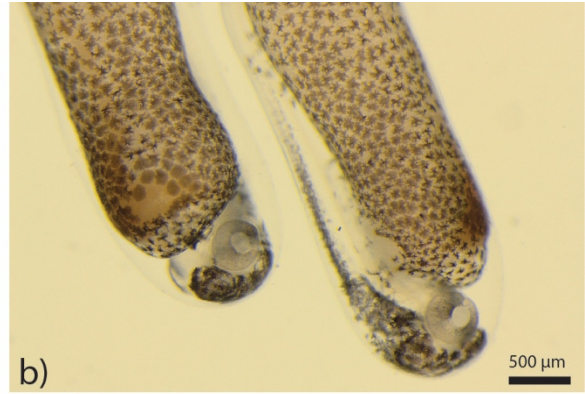
Appendix 2 Linear model output for $\dot{M}O_2$ in *Acanthochromis polyacanthus* and *Amphiprion melanopus* embryos. Boldness indicates significance *p*-values (α = 0.05).

Species	Factor	D.F.	*F*-value	*p*-value
Acanthochromis polyacanthus	Intercept	1, 344	1746.9047	**<0.0001**
	Temperature	1, 344	4.2698	**0.0395**
	Age	8, 344	31.3808	**<0.0001**
	Treatment	1, 344	2.7526	0.0980
	Mass	1, 344	13.5504	**0.0003**
	Age:Treatment	8, 344	2.1628	**0.0298**
Amphiprion melanopus	Intercept	1, 233	6872.852	**<0.0001**
	Age	5, 233	6.134	**<0.0001**
	Treatment	1, 233	3.416	0.0658
	Mass	1, 233	26.420	**<0.0001**
	Age:Treatment	5, 233	1.474	0.1992

Appendix 3 A Tukey multiple comparison test to find how $\dot{M}O_2$ changes with age in *Acanthochromis polyacanthus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance ($\alpha = 0.05$).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
2-1	0.86488	0.20838	4.151	**<0.001**
3-1	1.21110	0.21101	5.740	**<0.001**
4-1	1.91074	0.20978	9.108	**<0.001**
5-1	1.94113	0.22417	8.659	**<0.001**
6-1	1.86224	0.21986	8.470	**<0.001**
7-1	2.19553	0.21911	10.020	**<0.001**
8-1	2.07892	0.22544	9.222	**<0.001**
9-1	2.28219	0.23244	9.818	**<0.001**
3-2	0.34622	0.19692	1.758	0.70884
4-2	1.04586	0.19486	5.367	**<0.001**
5-2	1.07625	0.20865	5.158	**<0.001**
6-2	0.99736	0.20271	4.920	**<0.001**
7-2	1.33065	0.20520	6.485	**<0.001**
8-2	1.21404	0.20605	5.892	**<0.001**
9-2	1.41731	0.21728	6.523	**<0.001**
4-3	0.69963	0.19450	3.597	**0.00958**
5-3	0.73003	0.20802	3.509	**0.01301**
6-3	0.065114	0.20106	3.239	**0.03291**
7-3	0.098443	0.20472	4.809	**<0.001**
8-3	0.86782	0.20418	4.250	**<0.001**
9-3	1.07109	0.21550	4.970	**<0.001**
5-4	0.03039	0.20561	0.148	1.0000
6-4	-0.04849	0.19912	-0.244	1.0000
7-4	0.28480	0.20245	1.407	0.89512
8-4	0.16818	0.20165	0.834	0.99582
9-4	0.37145	0.21326	1.742	0.71938
6-5	-0.07889	0.21023	-0.375	0.99999
7-5	0.25440	0.21458	1.186	0.95945
8-5	0.13779	0.21282	0.647	0.99931
9-5	0.34106	0.22287	1.530	0.84073
7-6	0.33329	0.20744	1.607	0.80072
8-6	0.21667	0.20403	1.062	0.97930
9-6	0.41995	0.21139	1.987	0.55149
8-7	-0.11662	0.21037	-0.554	0.99978
9-7	0.08666	0.21993	0.394	0.99998
9-8	0.20327	0.21619	0.940	0.99058

Appendix 4 A Tukey multiple comparison test to find how $\dot{M}O_2$ changes with age in *Amphiprion melanopus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance ($\alpha = 0.05$).

| Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
2-1	0.01768	0.24955	0.071	1.0000
3-1	0.53781	0.25644	2.097	0.2872
4-1	0.62262	0.24538	2.537	0.1127
5-1	0.68618	0.25098	2.734	0.0682
6-1	0.60076	0.25201	2.384	0.1610
3-2	0.52013	0.22665	2.295	0.1951
4-2	0.60494	0.21564	2.805	0.0562
5-2	0.66850	0.22019	3.036	**0.0289**
6-2	0.58308	0.22186	2.628	0.0901
4-3	0.08481	0.22027	0.385	0.9989
5-3	0.14837	0.22373	0.663	0.9858
6-3	0.06295	0.22652	0.278	0.9998
5-4	0.06356	0.21448	0.296	0.9997
6-4	-0.02186	0.21694	-0.101	1.0000
6-5	-0.08542	0.22078	-0.387	0.9989

Appendix 5 A Tukey multiple comparison test to find where significant differences in $\dot{M}O_2$ lies within treatment and age in *Acanthochromis polyacanthus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Bold Pr(>|z|) values indicate significance ($\alpha = 0.05$).

Age (dpf) comparison		Estimate	Std. error	z-value	Pr(>|z|)
Control	Phenylhydrazine				
1	1	0.409340	0.231993	1.764	0.9528
2	2	0.113059	0.196523	0.575	1.0000
3	3	-0.071665	0.192512	-0.372	1.0000
4	4	-0.698438	0.199734	-3.497	**0.0491**
5	5	-0.238971	0.217845	-1.097	0.9998
6	6	-0.059157	0.199649	-0.296	1.0000
7	7	-0.197914	0.210294	-0.941	1.0000
8	8	0.056907	0.206598	0.275	1.0000
9	9	-0.324612	0.207659	-1.563	0.9856

Appendix 6 A Tukey multiple comparison test to find where significance lies within density of Ionocytes found along the trunk of embryos among ages in *Acanthochromis polyacanthus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
3-2	137.682	75.834	1.816	0.60128
4-2	247.236	75.834	3.260	**0.02413**
5-2	409.257	76.274	5.366	**<0.001**
6-2	156.875	75.893	2.067	0.42772
7-2	53.073	76.031	0.698	0.99693
8-2	20.476	74.986	0.273	0.99999
9-2	130.950	109.961	1.191	0.93231
4-3	109.554	68.755	1.593	0.74779
5-3	271.575	71.360	3.806	**0.00317**
6-3	19.193	69.290	0.277	0.99999
7-3	-84.608	69.442	-1.218	0.92404
8-3	-117.205	68.272	-1.717	0.66860
9-3	-6.732	106.643	-0.063	1.00000
5-4	162.021	71.360	2.270	0.30202
6-4	-90.361	69.290	-1.304	0.89404
7-4	-194.163	69.442	-2.796	0.09229
8-4	-226.760	68.272	-3.321	**0.01910**
9-4	-116.286	106.643	-1.090	0.95733
6-5	-252.382	71.042	-3.553	0.00883
7-5	-356.183	71.162	-5.005	**<0.001**
8-5	-388.780	70.786	-5.492	**<0.001**
9-5	-278.307	103.825	-2.681	0.12433
7-6	-103.802	69.605	-1.491	0.80685
8-6	-136.399	67.047	-2.034	0.44977
9-6	-25.925	106.447	-0.244	1.00000
8-7	-32.597	68.466	-0.476	0.99975
9-7	77.877	106.503	0.731	0.99589
9-8	110.474	106.888	1.034	0.96814

Appendix 7 A Tukey multiple comparison test to find where significance lies within density of Ionocytes found along the yolk-sac of embryos among ages in *Acanthochromis polyacanthus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
2-1	95.5690	44.6031	2.143	0.4358
3-1	119.0286	41.8880	2.842	0.1001
4-1	101.9281	43.0332	2.369	0.2938
5-1	205.2189	43.9789	4.666	**<0.01**
6-1	95.8914	43.3175	2.214	0.3885
7-1	115.7370	43.4974	2.661	0.1571
8-1	74.9915	42.9182	1.747	0.7099
9-1	218.0517	64.7880	3.366	**0.0211**
3-2	23.4596	41.8880	0.560	0.9998
4-2	6.3591	43.0332	0.148	1.0000
5-2	109.6499	43.9789	2.493	0.2286
6-2	0.3225	43.3175	0.007	1.0000
7-2	20.1680	43.4974	0.464	0.9999
8-2	-20.5775	42.9182	-0.479	0.9999
9-2	122.4827	64.7880	1.891	0.6123
4-3	-17.1005	40.150	-0.427	1.0000
5-3	86.1903	41.9041	2.057	0.4950
6-3	-23.1372	40.5158	-0.571	0.9997
7-3	-3.2916	40.7086	-0.081	1.0000
8-3	-44.0371	40.0790	-1.099	0.9735
9-3	99.0231	63.4111	1.562	0.8206
5-4	103.2908	42.7660	2.415	0.2683
6-4	-6.0367	41.6989	-0.145	1.0000
7-4	13.8089	41.8861	0.330	1.0000
8-4	-26.9366	41.2786	-0.653	0.9992
9-4	116.1236	63.9784	1.815	0.6645
6-5	-109.327	42.7025	-2.560	0.1981
7-5	-89.4819	42.8619	-2.088	0.4733
8-5	-130.227	42.7323	-3.048	0.0561
9-5	12.8328	62.3551	0.206	1.0000
7-6	19.8456	42.0588	0.472	0.9999
8-6	-20.8999	40.3218	-0.518	0.9999
9-6	122.1603	63.9524	1.910	0.5986
8-7	-40.7455	41.5540	-0.981	0.9871
9-7	102.3147	64.0392	1.598	0.8008
9-8	143.0602	64.3406	2.223	0.3816

Appendix 8 A Tukey multiple comparison test to find where significance lies within density of Ionocytes found along the trunk of embryos among ages in *Amphiprion melanopus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std.error	z-value	Pr(>|z|)
2-1	20.83	51.57	0.404	0.999
3-1	42.39	53.67	0.790	0.968
4-1	107.51	51.57	2.085	0.288
5-1	79.07	53.97	1.465	0.680
6-1	65.18	53.97	1.208	0.829
3-2	21.56	38.57	0.559	0.993
4-2	86.68	36.09	2.402	0.151
5-2	58.24	36.14	1.612	0.583
6-2	44.35	36.14	1.227	0.819
4-3	65.12	38.43	1.695	0.528
5-3	36.67	39.41	0.930	0.937
6-3	22.79	39.41	0.578	0.992
5-4	-28.45	37.42	-0.760	0.973
6-4	-42.33	37.42	-1.131	0.865
6-5	-13.89	34.94	-0.397	0.999

Appendix 9 A Tukey multiple comparison test to find where significance lies within density of Ionocytes found along the yolk-sac of embryos among ages in *Amphiprion melanopus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
2-1	-18.09	53.08	-0.341	0.999
3-1	54.95	59.35	0.926	0.940
4-1	-30.80	53.08	-0.580	0.992
5-1	35.13	53.08	0.662	0.986
6-1	98.11	53.08	1.848	0.433
3-2	73.04	59.35	1.231	0.821
4-2	-12.71	53.08	-0.239	1.000
5-2	53.22	53.08	1.003	0.917
6-2	116.20	53.08	2.189	0.242
4-3	-85.75	59.35	-1.445	0.699
5-3	-19.82	59.35	-0.334	0.999
6-3	43.16	59.35	0.727	0.979
5-4	65.93	53.08	1.242	0.815
6-4	128.91	53.08	2.429	0.146
6-5	62.98	53.08	1.187	0.843

Appendix 10 A Tukey multiple comparison test to find where significance lies within cell sizes of Ionocytes along the trunk of embryos among ages in *Amphiprion melanopus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
2-1	-35.425	27.469	-1.290	0.78301
3-1	-84.381	29.391	-2.871	**0.04485**
4-1	-68.009	27.427	-2.480	0.12455
5-1	-61.272	30.292	-2.023	0.31920
6-1	-148.093	30.292	-4.889	**<0.001**
3-2	-48.956	21.580	-2.269	0.19931
4-2	-32.584	19.263	-1.692	0.52608
5-2	-25.847	19.324	-1.338	0.75554
6-2	-112.668	19.324	-5.831	**<0.001**
4-3	16.372	21.187	0.773	0.97062
5-3	23.109	22.979	1.006	0.91239
6-3	-63.712	22.979	-2.773	0.05917
5-4	6.737	21.138	0.319	0.99954
6-4	-80.084	21.138	-3.789	**0.00198**
6-5	-86.821	17.838	-4.867	**<0.001**

Appendix 11 A Tukey multiple comparison test to find where significance lies within cell sizes of Ionocytes along the yolk-sac of embryos among ages in *Amphiprion melanopus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
2-1	54.72	29.45	1.858	0.4172
3-1	-47.72	44.44	-1.074	0.8870
4-1	-26.95	32.59	-0.827	0.9606
5-1	-105.91	32.12	-3.298	**0.0119**
6-1	-191.66	32.12	-5.968	**<0.001**
3-2	-102.44	44.44	-2.305	0.1847
4-2	-81.68	32.59	-2.506	0.1171
5-2	-160.63	32.12	-5.001	**<0.001**
6-2	-246.38	32.12	-7.671	**<0.001**
4-3	20.77	40.65	0.511	0.9955
5-3	-58.19	47.80	-1.217	0.8217
6-3	-143.94	47.80	-3.011	**0.0297**
5-4	-78.95	36.17	-2.183	0.2370
6-4	-164.7	36.17	-4.554	**<0.001**
6-5	-85.75	29.45	-2.912	**0.0398**

Appendix 12 A Tukey multiple comparison test to find where significance lies within cell sizes of Ionocytes along the yolk-sac of embryos among ages in *Acanthochromis polyacanthus*. Linear mixed effects models were used within the comparison test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
3-2	33.79	41.53	0.814	0.99198
4-2	10.61	42.45	0.250	1.00000
5-2	-100.14	46.43	-2.157	0.36900
6-2	-166.63	46.16	-3.160	**0.00699**
7-2	-389.10	54.72	-7.111	**<0.001**
8-2	-414.37	49.24	-8.415	**<0.001**
9-2	-257.37	65.09	-3.954	**0.00192**
4-3	-23.19	39.41	-0.588	0.99896
5-3	-133.93	45.70	-2.931	0.06352
6-3	-200.43	43.82	-4.574	**<0.001**
7-3	-422.89	52.15	-8.109	**<0.001**
8-3	-448.17	47.05	-9.525	**<0.001**
9-3	-291.16	64.58	-4.509	**<0.001**
5-4	-110.75	46.01	-2.407	0.22994
6-4	-177.24	44.75	-3.961	**0.00208**
7-4	-399.70	53.19	-7.515	**<0.001**
8-4	-424.98	47.93	-8.867	**<0.001**
9-4	-267.98	64.77	-4.138	**<0.001**
6-5	-66.49	48.90	-1.360	0.87024
7-5	-288.96	56.23	-5.139	**<0.001**
8-5	-314.23	50.80	-6.185	**<0.001**
9-5	-157.23	63.82	-2.464	0.20349
7-6	-222.46	56.67	-3.926	**0.00232**
8-6	-247.74	51.16	-4.843	**<0.001**
9-6	-90.74	67.61	-1.342	0.87790
8-7	-25.28	58.50	-0.432	0.99987
9-7	131.72	72.45	1.818	0.59715
9-8	157.00	68.98	2.276	0.29793

Appendix 13 A Tukey multiple comparison test to find where significance lies within cell sizes of Ionocytes along the trunk of embryos among ages in *Acanthochromis polyacanthus*. Linear mixed effects models were used within the comparisons test. Estimates and standard errors represent equally weighted means between each age group. Boldness indicates Pr(>|z|) values of significance (α = 0.05).

Age (dpf) comparison	Estimate	Std. error	z-value	Pr(>|z|)
3-2	-3.891	20.846	-0.187	1.00000
4-2	-62.360	20.846	-2.992	0.05332
5-2	-80.749	20.798	-3.883	**0.00246**
6-2	-53.402	21.071	-2.534	0.17415
7-2	-72.301	21.997	-3.287	0.02156
8-2	-90.369	22.143	-4.081	**0.00102**
9-2	-39.645	28.034	-1.414	0.84531
4-3	-58.469	16.102	-3.631	**0.00659**
5-3	-76.858	16.178	-4.751	**<0.001**
6-3	-49.511	16.441	-3.011	0.05062
7-3	-68.410	17.607	-3.885	**0.00251**
8-3	-86.478	17.679	-4.891	**<0.001**
9-3	-35.754	25.148	-1.422	0.84172
5-4	-18.389	16.178	-1.137	0.94646
6-4	8.959	16.441	0.545	0.99937
7-4	-9.941	17.607	-0.565	0.99921
8-4	-28.009	17.679	-1.584	0.75210
9-4	22.715	25.148	0.903	0.98508
6-5	27.347	16.443	1.663	0.70249
7-5	8.448	17.653	0.479	0.99973
8-5	-9.620	17.781	-0.541	0.99940
9-5	41.104	24.795	1.658	0.70612
7-6	-18.899	17.831	-1.060	0.96313
8-6	-36.968	17.974	-2.057	0.43336
9-6	13.757	25.226	0.545	0.99937
8-7	-18.068	10.096	-0.946	0.98050
9-7	32.656	26.016	1.255	0.91118
9-8	50.724	26.397	1.922	0.52518