How Do Coral Reef Fishes Develop into Athletes?

Thesis submitted by

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“The difficulty and cost of working directly with larval reef fishes has been one reason for the focus of studies on settlement and recruitment, and we know far more about the ecology of reef fish in the two weeks following settlement than in the two weeks prior to that event.”

-Peter Sale

Acknowledgements

To be honest, it is very hard to put into words the past four years of my life, and what doing this PhD has taught me. However, what is more tangible is that through this opportunity I had the absolute privilege to work with several amazing people, whom I hope I will work with throughout my career. More importantly than the science, the following people are all incredibly genuine people whom I have learned a lot from, and allowed me to grow as human being.

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Statement of Contribution of Others

The following thesis represents a collaborative effort between myself and my advisory committee A/Prof Jodie Rummer (Primary Advisor), Prof. Mark McCormick (Advisor Mentor), Dr. Peter Cowman (Secondary Advisor), and Dr. Björn Illing (Secondary advisor) all of whom are based at the Australian Research Council Center of Excellence (ARC CoE) for Coral Reef Studies at James Cook University (JCU) Townsville campus. I was fortunate enough to collaborate with numerous researchers outside of my committee, whose names, affiliations and roles are listed below. Despite this collaborative effort, I was responsible for designing all aspects of my thesis (e.g., experimental design), animal husbandry, experimentation, data collection/analysis/interpretation and writing of this thesis document.

My candidature was supported by an International Research Training Program Stipend from James Cook University. I received research support from my advisor (JLR) whom is supported by an ARC (Discovery Early Career Research Award, PDE150101266), the L'Oreal-UNESCO Women in Sciences Foundation, and the ARC Centre of Excellence for Coral Reef Studies. Additionally, I received research funds from the ARC Center of Excellence (David Yellowlees Award) and the Australian Society for Fish Biology.

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The roles of all collaborators who contributed are listed below for each chapter of this thesis. Each individual is recognized for their contributions in their respective thesis chapter, as well as in any published or future peer-reviewed papers.

Chapter 1. General introduction

Conceptualization: ATD, JLR, MIM, PFC, BI; Writing (first draft): ATD; Writing (review & revision): ATD, JLR, MIM, PFC, BI

Chapter 2. Swimming performance of marine fish larvae: review of a universal trait under ecological and environmental pressure

Conceptualization: ATD, Björn Illing (ARC CoE), Ana Faria (MARE-ISPA), JLR; Literature search: ATD, BI, AF; Data analysis: ATD, BI; Writing (first draft): ATD; Writing (review & revision): ATD, BI, AF, JLR.

Chapter 3. Habitat association may influence swimming performance in marine teleost larvae

Conceptualization: ATD, PFC, Jeffery M Leis (University of Tasmania), MIM, JLR; Literature search: ATD; Data Analysis: ATD, PFC; Writing (first draft): ATD; Writing (review & revision): ATD, JML, PFC, MIM, JLR

Chapter 4. Muscle Tissue Development of a Coral Reef Fish Species during embryonic and larval stages

Conceptualization: ATD, JLR, MIM, PFC, BI; Experimentation and Histology: ATD, BI, Lit Chein Cheah (JCU); Writing (Intro and Methods): ATD; Funding: JLR

Chapter 5: The Exercise Physiology of Larval Reef Fishes Over Ontogeny

Conceptualization: ATD, JLR, PFC, MIM, BI, Sjannie Lefevre (University of Oslo), Göran Nilsson (University of Oslo); Animal Husbandry: ATD, Michael Jarrold (JCU); Experimentation: ATD; Data analysis: ATD; RNA extractions: Jessica Harris (JCU), ATD; Bioinformatics: SJ; Writing (first draft): ATD; Writing (review & revisions): ATD, JLR, MIM, PFC, BI; Funding: JLR, ATD, GN

Chapter 6: The Physiological Mechanisms Underpinning Metamorphosis in Reef Fishes

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Chapter 7: General Discussion

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General abstract

The way animals move through their environment provides critical information as to their ability to respond to environmental stress, interact with conspecifics and heterospecifics simultaneously to acquire energy while avoiding becoming 'acquired energy', and undertake seasonal migrations. For many animals, like fishes, the ability to move develops during early life history stages. Tropical coral reef fishes, while previously perceived as poor swimmers as larvae, are actually capable of impressive swimming abilities during early ontogenetic stages, which ultimately influences their ability to disperse and successfully recruit to coral reefs. Despite being anecdotally referred to as 'athletes', we know very little about how coral reef fishes physiologically support such performance. This thesis investigates large-scale patterns in swimming performance in larval fishes, how reef fishes develop the ability to swim, the energy required to support swimming in reef fishes, and the physiological mechanisms that enable reef fishes to successfully recruit to reefs.

In **chapter 2** I provide an overview of how anatomical structures (e.g., muscles fins and gills) develop over ontogeny of marine fishes, describe laboratory and field techniques that measure swimming ability and their ecological relevance, and undertake a meta-analysis on how different environmental stressors (e.g., temperature, prey-reductions) impact swimming across developmental stages and latitude. Looking at the current larval fish swimming literature yielded several important findings. First, there is a clear bias toward studying swimming in older larval stages (e.g., post-flexion) of fishes, and I suggest that future work should investigate the swimming ability of pre-flexion larvae, especially when exposed to environmental stressors. The need to look at the entire larval duration set the foundation for **chapter 5**'s methodology. Second, endurance swimming, the ability to swim long distances, was most impacted by environmental stress, particularly prey reduction. Thirdly, I determined that high swimming capabilities were found in fishes from tropical latitudes, but these findings may be masked due to environmental temperatures. To tease apart performance from latitude (i.e., tropical vs. temperate species), I compared the swimming performance of two orders of fishes – Perciformes, which encompass most reef fishes, and Clupeiformes, which are the herrings – on which data are found across common latitudes and ontogenetic stages. I found that swimming performance between these two groups is similar at temperate and tropical latitudes. The similarities in swimming performance between tropical Clupeiformes and Perciformes brings to light interest in large scale differences in swimming performance between groups of fishes across latitude as well as habitat, which initiated the hypotheses of **chapter 3**, in that habitat selection may influence swimming performance of marine fish larvae.

The aim of **chapter 3** was to use a phylogenetic comparative analysis, meta-analysis, and case study approach to investigate patterns in marine fish swimming performance across ontogeny, habitat (reef, non-reef demersal, and pelagic), and latitude (temperate and tropical). I found that temperate reef fishes have the highest increase in swimming performance with growth, and swim at similar capacities to tropical demersal and pelagic fishes, despite the 10°C difference in water temperature. Tropical reef fishes generally have the fastest

swimming performance compared to other groups regardless of latitude, even when adjusted for size, and likely need to grow larger to outswim high reef currents. The results from **chapter 3** suggests that, for fish larvae, the need to find a patchily distributed coastal reef requires large body sizes to achieve higher swimming abilities than simply remaining in the pelagic environment or finding a random benthic habitat on which to settle. The clear functional importance of large body sizes during the larval period provides ample context for **chapter 4**, which investigates the muscle development of reef fish embryos and larvae.

During early life, most of a larval fish's energy is diverted toward muscle development to improve swimming. In **chapter 3**, I found that reef fishes are larger at any stage of development than pelagic and non-reef demersal fishes, and I therefore predicted that tropical reef fishes must have rapid muscle tissue synthesis to support high swimming ability. I designed **chapter 4** to histologically assess muscle fiber growth and development in an anemonefish (*Amphiprion melanopus*) over embryonic and larval stages and compare muscle fiber growth rates to those of other groups of fishes. Unfortunately, due to complications arisen from COVID-19, this chapter was omitted from the formal thesis; however, this topic remains an important link between the previous chapter and the following chapter on swimming energetics. If reef fishes have predictably higher rates of tissue synthesis in order to swim at high capacities, their metabolism must match the demand to support these physiological processes.

High rates of tissue synthesis (predicted from **chapter 4**) to achieve large body sizes (**chapter 3**), and impressive swimming speeds (based on literature analyses from **chapters 2** and **3**) must be supported from predicted high oxygen uptake rates. Additionally, during the settlement transition period reef fishes must transition from the oxygen saturated pelagic environment (larval stage) to the reef (juvenile and adult phases), which can be oxygen limited (hypoxic) in some nursery habitats at night from coral respiration. Therefore, it is possible that oxygen demands and how oxygen is delivered to tissues changes over ontogeny to prepare reef fishes for the transition between the distinct conditions of the pelagic and reef habitats. In **chapter 5**, I investigated the oxygen uptake rates that support swimming performance of a reef fish species (cinnamon anemonefish, *Amphiprion melanopus*) over the entire larval phase. Further, I also investigated the potential ontogenetic shifts in haemoglobin and myoglobin gene expression, proteins that transport and deliver oxygen to tissues respectively, that may facilitate the transition between pelagic and reef habitats. I found that reef fishes support high performance swimming through high oxygen uptake rates (100,000-20, 000 mg O_2 kg^{-1} h^{-1}), with higher rates than any other teleost in the published literature. The expression of haemoglobin and myoglobin also increased during the onset of reaching the reef, suggesting how oxygen is delivered to the tissues is different during the pelagic phase than reaching a benthic reef home. **Chapter 5** shows that oxygen demands and the mechanisms that deliver oxygen change during the pelagic larval phase before they reach a reef. This transition is important and is also complimented by several physiological changes, as shown by **chapter 6**.

The pelagic-to-reef transition event is considered a population bottleneck in reef fish life history due to the high predation rates experienced by newly-settled fishes. Because of different selection pressures in the two environments, reef fishes must undertake a rapid, species-specific change in morphology and physiology, known as metamorphosis, to successfully recruit to reefs. However, little is known of the physiological changes over this critical period. In **chapter 6**, I investigated changes in the swimming capabilities, respiratory metabolism, and muscle tissue energetics (i.e., mitochondria volume densities) over metamorphosis for a common damselfish (Ambon damselfish, *Pomacentrus amboinensis*). Swimming performance decreased by 40% after 72 h post-settlement, which may be a behavioural response to save energy for growth. Muscle mitochondria volume densities increased by 20% upon settlement to the reef which, based on lower cost of transport estimates at this stage, may be what enables these reef fishes to swim efficiently. These densities decrease a few days after settlement. These results show that metamorphosis involves fundamental changes in energy budgets and muscle properties as fish transition from being mobile pelagic predators to more sedentary reef-associated planktivores.

The research here is among the first to investigate the oxygen demands that support swimming during the pelagic larval stage of tropical reef fishes. These processes have critical impacts on dispersal and connectivity among reefs, as well as successful recruitment to reefs. Understanding the physiological mechanisms underpinning swimming performance allows for an improved understanding of how reef fishes support high swimming capabilities that ultimately allows them to find and settle onto a reef. These mechanisms integrate together at multiple levels of biological organization – whole animal, tissue, cellular and molecular – and therefore the multidisciplinary approach taken by this thesis provides a more complete mechanistic understanding as to how reef fishes develop into amazing athletes. Future work should broaden the taxonomic range of species investigated, especially in terms of swimming performance of temperate species and swimming energetics of late-stage tropical reef fishes (e.g., beyond damselfishes) and temperate reef fishes. Additionally, understanding how energy is portioned to growth and swimming will be invaluable for bioenergetics and individual-based models for tropical species in order to better understand the relationship between swimming and hydrodynamic processes. Overall, this research highlights the complexities of understanding the development and energetics of swimming performance of fish larvae and how, at such a young age, tropical reef fishes support their critically important athletic capabilities.

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Chapter 1. General Introduction

1.1 Performance and athleticism among animals

Performance can be defined as any physically demanding task that an animal expends energy in order to interact within their environment, conspecifics, or heterospecifics (Irschick & Higham, 2013). These interactions include, but are not limited to, predator-prey interactions, moving through the environment (e.g., seasonal migrations and/or daily movements) and avoiding suboptimal conditions (e.g., seasonal and/or daily variations in temperature). Indeed, many of these relevant interactions are critical for the survival of an organism, resulting in competition among species for resources, such as food and territory. However, over evolutionary history, some species have specifically developed physical traits to provide them with an advantage that enables them to outcompete other species for resources. Animals that are capable of functioning at a higher capacity for any given metric of performance than average performers are categorized as 'athletic' (Sharp, 2012). This definition of athleticism involves interspecific (i.e., between species) differences in performance between animals, and will be the focus of my thesis, as I compare swimming performance between the larvae of different species of fishes (specifically comparing tropical reef-associated species with pelagic and non-reef demersal fishes). However, it is important to note that athleticism can also be related intraspecifically (i.e., among species), as individuals within a given population with higher performance capabilities (e.g., faster sprinting speeds) have a higher chance of survival and passing their genes onto the next generation. Therefore, the advantage of athletic performance is to occupy a very specific ecological niche and thus access specific resources (Irschick & Higham, 2013). Athleticism is a wide-spread phenomena across the animal kingdom, including mammals (e.g., sprinting speed of the cheetah *Acinonyx jabatus,* Flindt, 2006), birds (11,000 km non-stop migration route from Alaska to New Zealand of the bar-tailed godwit *Limosa lapponica*, Gill Jr. et al, 2005), reptiles (leatherback turtles *Dermochelys coriacea* are capable of diving up to 1,230m, Hays et al., 2004), amphibians (high active metabolic rates of poison dart frog species from Family Dendrobatoidea, Santos, 2012), invertebrates (leafcutter ants, species from the genera Atta and Acromyrmex, can lift 50 times their body weight with their jaws, Balamonica et al., 2019), and the focus

of my thesis, fishes (specifically the high speed swimming of larval tropical reef fishes, Stobutzki and Bellwood, 1994).

Athleticism offers a unique perspective on performance, due to the specializations in morphology, anatomy, and physiology required to function at a level above other species (Irschick & Higham, 2013). Within this context, physiology relates to the mechanistic processes (e.g., oxygen uptake rates) that support and a specific performance metric. Most animal athletes, when compared to less-athletic species, have some or all of the following physiological and anatomical features: greater skeletal muscle mass (Weibel et al., 2004), a larger heart (Bishop, 1997), increased respiratory surface area (Muir & Hughes, 1969), increased tissue maintenance associated with a greater expenditure of ATP (i.e., adenosine triphosphate) during high performance activity (Killen et al., 2010), and increased muscle mitochondrial density, which is correlated with increased total energy expenditure (Hoppeler et al., 1987). In general, athletic performance is highlighted in the context of predator-prey interactions where, for example, a cheetah (*Acinonyx jabatus*) can capture fast prey that other animals cannot catch (Flindt, 2006) and migratory behaviour where, for example, Sockeye salmon (*Oncorhynchus nerka*) undergo annual migrations spanning thousands of kilometres to exploit seasonal changes in food abundance and spawn in ideal habitats (Quinn & Dittman, 1990). Understanding how traits/anatomical structures that are associated with high performance grow and develop over the life-history of an organism provides important information regarding energy utilisation and ontogenetic changes in life-style.

1.2 Swimming performance of larval fishes

Organisms are generally at their most vulnerable during the earliest stages of development. In particular, the larval stage of marine fishes is characterised by high mortality rates attributed to the high energy demands associated with growth and avoiding predation (McGurk, 1986; Bailey & Houde, 1989; Houde 1989, 1994). Indeed, early life is difficult for fish larvae, regardless of latitude (e.g., temperate, tropical, or polar), since larvae are small at hatch, and they lack the swimming capabilities to avoid

predators and capture food. Accordingly, there has been much interest in understanding the larval phase of fishes, as larval survival is important for maintaining adult populations.

Most marine fishes have a dispersive, planktonic larval phase (Thorson, 1950). Eggs, laid either in the open ocean (e.g., pelagic fishes) or on benthic substrate along the coast (e.g., some reef fishes, mainly damselfishes, Pomacentridae) are carried by oceanic currents. Larvae hatch from eggs and develop (e.g., growth of tissues such as organ systems, muscles, and fins) in the open ocean. As such, the Simplifying Assumption (SA) has been previously used to characterise movement patterns of fish larvae and thus used to parameterise classic dispersal models (Frank & Leggett, 1982). The characteristics of the SA are as follows: I) larvae passively drift with currents due to poor swimming capabilities; II) the length of the pelagic larval duration is the only biological variable that impacts dispersal; III) larval behaviour is homogenous within and among species regardless of geographic location; and IV) larvae do not choose their settlement location, but rather currents disperse larvae along a predictable hydrodynamic path until the end of their respective larval phase (Frank & Leggett, 1982; Roberts, 1997). While these assumptions were previously believed to characterise the larval phase for all fishes, research since the early 1990s has shown that there are species-specific differences in the extent to which larvae adhere to these important assumptions (Leis, 2006).

Early evaluations of swimming performance of larval fishes primarily focused on temperate fish families, such as cods (Gadidae), herrings (Clupeidae), and flatfishes (Pleuronectiformes), mainly due to their importance for commercial fisheries (Miller et al., 1988; Leis, 2006). Temperate fish larvae develop swimming structures, such as the caudal fin (i.e., this landmark event is known as flexion), at 16-20 mm total length (TL; Leis & McCormick, 2002); however, temperate fish larvae at this stage are incapable of any significant horizontal movements against ocean currents (known as influential swimming) and require further somatic growth of muscle mass to enable such improved swimming performance (Blaxter, 1988; Miller et al., 1988). At flexion-stage sizes, temperate fishes (i.e., even over a wide taxonomic range) swim, on average, at speeds slower than 5cm sec^{-1}; their burst swimming (e.g., for predator avoidance or

prey capture) is slower than 25 cm sec^{-1} (Miller et al., 1988), which are speeds typically not faster than ambient currents, and are therefore too slow to influence dispersal, thus, classify these groups of fishes at this developmental stage as planktonic or passive particles. This highly contrasts the swimming capabilities for tropical coral reef fishes.

Coral reef fish larvae begin flexion at smaller sizes (3-5mm TL; Mccormick & Leis, 2002) than temperate fishes and were therefore previously classified under the SA (Roberts, 1997). This assumption has important implications for coral reef fishes, as they are site-specific as adults and thus rely on larvae for dispersal and expanding habitat ranges. The vast majority of reef-associated fish life history involves a larval stage that develops in the open ocean, a few kilometres away from the reef (reviewed by Leis & Fisher, 2006). Ocean currents carry the developing embryos away from the reef and into the pelagic environment where the larvae hatch, grow, and develop until they return to the reef to settle. The length of time in the pelagic zone is called the pelagic larval duration (PLD), and it was previously assumed that during this period, passive dispersal of larvae via ocean currents carries larvae back to the reef (Roberts, 1997). Up until the early 1990s the extent to which reef fish populations were connected to one another was unknown, as dispersal was assumed to be reliant on currents (Leis, 2006; Roberts, 1997). Genetic parentage analysis and tagging techniques have now revealed a variable, but surprisingly high level, of recruitment back to natal home ranges, known as self-recruitment (Jones et al., 1999; 2005). Given the small size and rapid development of fish larvae, these findings have astounded many scientists, prompting questions regarding how larvae manage to achieve these so-called feats of athleticism. Luckily, a variety of laboratory and field approaches have enabled the exercise physiology of larvae to be assessed, which promises a better understanding of the ecology of coral reef fish larvae in the near future.

1.3 The exercise physiology of tropical coral reef fish larvae

Several key studies have advanced our understanding of the exercise physiology of coral reef fish larvae and specifically have demonstrated that reef fish larvae are remarkably athletic swimmers, thus refuting the notion that coral reef fish larvae fit under the SA. The first study to evaluate the swimming

capabilities of coral reef fish larvae was by Stobutzki & Bellwood, (1994); they used a multi-channelled swim tunnel to evaluate the swimming speeds of pre- and post-settled larvae (Ambon damselfish, *Pomacentrus amboinensis*; *Dischistodus sp.*; Chinese demoiselle, *Neopomacentrus bankieri*). These damselfish larvae are capable of high speed, sustained swimming (38.2cm sec^{-1} and 26.3cm sec^{-1} for pooled pre- and post-settled larvae, respectively; Stobutzki & Bellwood, 1994). Additionally, Fisher & Bellwood, (2001) found that pre-settlement anemonefish (*Amphiprion melanopus*) larvae are also capable of long distance endurance swimming, as fed larvae were capable of swimming for 28.7km before fatigue at a water velocity of 7 cm sec^{-1}. High swimming capacity was not only observed in damselfishes. Fisher et al., (2005) noted high swim performance over a wide taxonomic range of reef-associated families, including Chaetodonidae (48.8±2.3 cm sec^{-1}), Acanthuridae (50.5±1.4 cm sec^{-1}), and Siganidae (67.1±8.9 cm sec^{-1}), among several other families. The high swimming capacity of reef fish larvae was validated using *in situ* observations in the field, which determined the average swimming speed across a broad range of reef associated families was 30 cm sec^{-1} and ranged from 6.3cm sec^{-1} to 65.5cm sec^{-1} (Leis et al., 1996; Leis & Carson-Ewart, 1997, 1998, 1999, 2001). Even at hatch (1.4-4.5 mm TL), some coral reef fish larvae can achieve speeds of 2 cm sec^{-1} (4.4-14.2 BL sec^{-1}), with maximum speeds of 4 cm sec^{-1} (14 BL sec^{-1}) (Fisher, 2005; Leis, 2010). The average swimming speeds of tropical reef fish larvae are as fast as the burst swimming speeds of temperate larvae (Williams et al., 1996), and therefore, these field observations further support that reef fish larvae can swim faster than ambient currents around Lizard Island (13.5 cm s^{-1}; Leis, 1986) and thus have direct control over their distribution (Leis & Carson-Ewart, 1997). These findings for coral reef fish larvae contrast with their temperate species counterparts. Batty, (1994) found that Atlantic herring (*Clupea harengus*) larvae (2 cm TL) could only swim 0.5-6 cm sec^{-1} (0.25-3 BL sec^{-1}), more than an order of magnitude slower than coral reef fish larvae. The sardine (*Sardina pilchardus*) must grow to 19.1mm TL (55dph; Silva et al*.,* 2014) in order to achieve influential swimming (9.47cm sec^{-1}). Indeed, when taken together, laboratory and *in situ* studies reveal that coral reef fish larvae are capable of high calibre swimming performance, and such behaviours have critical ecological consequences on dispersal and connectivity.

1.4 Why does swimming performance matter for coral reef fish ecology?

Much of the current understanding of the swimming capabilities of coral reef fish larvae originates from attempting to answer a fundamental ecological question regarding whether coral reef fish populations are open (i.e., reliant on an external supply of larvae) or closed (i.e., self-replenishing) systems (Armsworth et al., 2001; Armsworth, 2000, 2001, 2002; Mora & Sale, 2002; Sale, 2004). This question is centred around the concept of 'connectivity', which is defined as the extent to which individual populations are linked by dispersal at the larval, juvenile, and adult stages (Saenz-Agudelo et al., 2011). For large reef ecosystems such as the Great Barrier Reef, which spans 344, 400 km^2 and comprised of 3000 individual reefs (<1-50km separation between individual reefs; Almany et al., 2007, 2009, 2013), understanding connectivity has important implications for fisheries and marine protected area (MPA) management (Sale, 2004; Harrison et al., 2012). Since larvae of most marine species, including coral reef fishes, were considered passive under the SA, coral reef ecosystems were considered to be open, as biophysical oceanographic models showed how currents are capable of dispersing larvae over large distances (Roberts, 1997). However, for coral reef fishes, evidence for self-recruitment to patch reefs began to emerge (Jones et al., 1999). Moreover, technological advancements allowed for genetic subdivision of species to be determined. The evidence suggesting that reef fish populations are capable of self-recruiting to natal reefs became a crucial discovery with important implications for management and for the field of exercise physiology.

Since start of the 21st century, there has been a focus on the recruitment strategies of coral reef fishes and the extent to which individual populations on isolated reefs (i.e., metapopulations) are capable of self-recruitment. Advances in technology and new techniques, such as tetracycline markings on otoliths (Jones et al., 1999, 2005), otolith chemistry (Swearer et al., 1999), and DNA parentage analysis (Planes et al., 2009) shifted to the forefront of this research. Jones et al., (1999) used tetracycline to mark the otoliths of Ambon damselfish (*Pomacentrus amboinensis*) and estimated that 15-60% of juveniles are returning to their natal reefs. Subsequently, Jones et al., (2005) utilised a similar technique on saddleback clownfish

(*Amphiprion polymnus*) and found that 33% of larvae returned to within 2 hectares of their parent anemone, and 5 individuals returned to within 50 meters of their parents. However, self-recruitment of coral reef fish larvae is highly variable, both spatially, as well as among species. For example, self-recruitment of neon damselfish (*Pomacentrus coelestis*) averages 75% on Lizard Island (Patterson et al., 2005), the wrasses' (*Coris bulbifrons* and *C. picta*) population on Lord Howe Island experienced a 26-65% self-recruitment rate (Patterson & Swearer 2007), and the Papua New Guinea metapopulation of *A. polynmus* exhibits an 18% self-recruitment rate (Saenz-Agudelo et al., 2011). These examples, among many others, provide compelling evidence suggesting that self-recruitment is occurring on reefs, but what does this mean for coral reef ecosystems?

Not only does evidence for self-recruitment of coral reef fishes provide new insights into the early life history of reef-associated fishes from a behavioural and physiological perspective, but this information can potentially change how reefs are managed as well. Coral reefs are now considered, spatial scale-dependent, open systems comprised of semi-closed metapopulations (Mora & Sale, 2002; Sale, 2004; Christie et al., 2010; Saenz-Agudelo et al., 2011) . Fisheries and MPA management have benefited from understanding self-recruitment strategies of reef fishes. For example, over three marine protected reserves, 83% of coral trout (*Plectropomus maculatus*) and 55% of stripey snapper (*Lutjanus carponotatus*) larvae were exported to adjacent, fished reefs, with the remaining larvae self-recruiting to their natal reefs (Harrison et al., 2012). Additionally, self-recruiting strategies retain local genetic diversity, improving the species' capacity to adapt to environmental change (Taylor & Hellberg, 2003; Patterson & Swearer, 2007). While the benefits and overall 'strategy' of coral reef fish dispersal is gradually becoming more apparent, the mechanism underlying dispersal is still unknown. Few of the mentioned examples acknowledge larval behaviour, either by some sensory mechanism or via swimming performance, as factoring into self-recruitment or dispersal models. While this provides an ecological context for dispersal strategies, there are many questions regarding the mechanisms supporting

performance and to what extent reef fish larvae use their swimming capabilities to stay close to or move away from their natal reef (Cowen et al., 2000).

1.5 Critical knowledge gaps

According to (Sale, 2004), we know more about the biology and ecology of coral reef fishes two weeks after settlement than during the entirety of their PLD. To this date, nearly 20 years later this statement still holds true. Thus, physiological and anatomical traits of coral reef fishes, during early ontogeny and throughout their PLD, represents an important knowledge gap that must be understood not only for knowledge advancement regarding the ontogeny of important functions and structures, such as feeding and swimming, but also to provide valuable information for population dynamics, dispersal, and recruitment strategies. While swimming performance methodologies (e.g., critical swimming speed, and endurance tests) and *in situ* observations have established that coral reef fish larvae have a high capacity for swimming, the extent to which they utilise their swimming for dispersal and the physiological mechanisms powering such performance represent an area of particular interest. This thesis has been designed to address some of these knowledge gaps, including the phylogenetic differences in swimming performance between groups of fishes during early life history, the ontogeny of muscle tissue development, the energy demands that support growth and swimming over ontogeny, and the physiological aspects of metamorphosis.

The swimming performance of different fish groups during early life history is clearly highly variable and also influenced by many biotic and abiotic factors. While it has been already established that, as larvae, tropical reef fishes are superior swimmers when compared to temperate fishes, questions still remain as to the factors that contribute the most to these different performance patterns. Including habitat selection (e.g., reef and non-reef association) into comparisons may reveal drivers of performance, especially when comparing across latitude. Does reef association influence swimming performance regardless of latitude? Additionally, no studies have placed performance into a phylogenetic comparative framework to investigate how species relatedness may contribute to performance. Such analyses may

reveal how multiple factors influence swimming performance during early life history, such as aspects of physiology and anatomy, including muscle tissue development.

Most studies investigating muscle tissue development in fishes have been on species from temperate fish groups, including Clupeidae (Vieira & Johnston, 1992; Johnston, 1993) and Cyprinidae (El-Fiky et al., 1987; El-Fiky & Wieser, 1988). But it is important to note that swimming muscles generally develop in concert with other organ systems, such as the gills, and the fins (Downie et al., 2020). All temperate larvae that have been studied to date hatch with a superficial layer of red fibers used for gas exchange; this under-developed muscle tissue later develops into adult red muscle. These temperate larvae also hatch with undifferentiated myomeres that eventually develop into adult white muscle (Johnston & McLay, 1997; Johnston, 1999). Muscles do not become 'fully developed' until the larvae reach a critical size, which is also usually around the time when gills form, because the larvae must be large enough to accommodate larger muscle blocks for swimming (Vieira & Johnston, 1992; Nathanailides et al., 1995; Wells & Pinder, 1996; Osse et al., 1997). Currently it is unknown whether this developmental pattern occurs in tropical species as well. Ramírez-Zarzosa et al., (1995) found that the sub-tropical sea bass (*Dicentrarchus labrax*) begin developing adult red and white muscle fibers at 4-5 dph (days post hatch), and Johnston, (1993) found that rainbow trout (*Oncorhynchus mykiss*), a temperate fish, do not possess adult red muscle fibers until 50 dph. In contrast, Prescott et al., (in review) found that two demersal egg-laying tropical species (i.e., a damselfish and an anemonefish) develop their gills prior to hatching. This unique developmental pattern is vastly different than that of other teleost fishes and may infer that, for these species, muscle tissue develops differently as well, given the relationship between gill and muscle development during early ontogeny. Currently, muscle development of tropical coral reef fishes is not well-understood, but investigating how muscle develops may reveal how tropical fishes support swimming performance relevant for dispersal at such an early age. If adult red muscles are present at hatch, or very early in ontogeny, larvae will need to supply these highly metabolically active

tissues with oxygen, and thus a further understanding of energy demands that support swimming and tissue function will also be highly important.

Compared to adult fishes, larvae have received less attention with respect to researching the oxygen demands to support swimming (Peck & Moyano, 2016). Larvae have higher oxygen uptake rates than adult conspecifics, on a mass-specific basis, to support the high growth rates characteristic of this developmental stage (Post & Lee, 1996). Thus, it has been suggested that there is less oxygen available for other ecologically relevant tasks, such as performance and responding to environmental stress (Killen et al., 2007). Peck & Moyano, (2016) reported that 59 studies had investigated marine fish larvae oxygen consumption rates; most of the studied focused on temperate (gadoids comprised 39% of the study species) and subtropical (49%) fish species. Only 9% of the studies focused on the oxygen demands of tropical fish larvae (Peck & Moyano, 2016). Additionally, the vast majority (95%) of research at this time had focused on routine metabolic rates (e.g., oxygen uptake of unprovoked individuals). Only four studies have investigated active metabolic rates (i.e., fish under exercise scenarios) which may be more indicative of the oxygen demands to support swimming. At the time of the Peck and Moyano review (2016), no studies had focused on the oxygen uptake during activity of larval coral reef fishes at any stage of development, let alone attempted to understand energy budgets over early life history. Furthermore, there is a general lack of understanding as to whether the mechanisms underpinning transporting (e.g., haemoglobin gene expression) and delivering oxygen to tissues (e.g., myoglobin gene expression) changes over larval development. Specifically, as larvae improve their swimming abilities, does haemoglobin gene expression change its ability to transport oxygen accordingly? Understanding oxygen demands over early ontogeny provides the best insight into the interactive metabolic costs associated with growth, performance, and basic maintenance during a critical life history stage. A perfect balance between the costs and benefits enable larvae to survive their pelagic stage and find a suitable reef habitat on which to settle.

Metamorphosis marks the transition between pelagic and reef stages and is considered a critical bottleneck in coral reef fish life history due to high selective predation-associated mortality. Yet for such a critical life history milestone, we know very little about the physiological traits that enable reef fishes to successfully settle onto reefs. An emerging pattern associated with reef fish metamorphosis, albeit based on a small number of studied species (e.g., pomacentrids and labrids; Leis et al., 2011), is the decline in athletic capacity immediately following settlement. While this has been suggested as a mechanism to cope with nocturnal hypoxic conditions on the reef (Nilsson et al., 2007a,b) and/or to enhance maneuverability among a structurally complex habitat (Leis et al., 2011), it is not yet clear what mechanism is responsible for this drastic change in performance. For example, does muscle composition change? Or, are there changes in oxygen delivery to muscle tissues (e.g., changes in muscle mitochondrial density)? Some studies have noted changes in muscle composition as pelagic larvae transition to benthic juveniles/adults (e.g., gadiformes; Finn et al., 2002). Could the same developmental pattern occur in tropical reef fishes? If so, such re-organization and alterations to swimming tissues may come with a metabolic cost, potentially limiting performance and thus leaving newly settled larvae vulnerable to predation. Therefore, a better mechanistic understanding as to how reef fishes successfully transition from pelagic to reef habitats may lead to a better understanding of recruitment strategies, adult population structure, and general population dynamics of reef fishes.

1.6 Research aims and thesis outline

Given the current knowledge gaps, this thesis takes a multi-disciplinary approach to further the understanding of the development, evolution, ecology, and physiology of coral reef fishes during early ontogeny. Specifically, I will combine laboratory and field work, encompassing swimming respirometry, histology, and phylogenetic comparative analysis of exercise performance and tissue development metrics for coral reef fishes. My thesis tests the hypothesis that coral reef fishes develop in the ‘physiological extreme’, requiring high amounts of oxygen to support rapid tissue synthesis within their short larval durations to support fast swimming, and these requirements are mediated by changes at the tissue, cellular

and genetic level. To test this overarching hypothesis, my thesis' chapters investigate the following hypotheses:

Chapter 3: Reef association influences strong swimming performance among fish larvae, versus remaining in the pelagic environment or finding a random benthic patch on which to settle.

Chapter 4: Focal tropical coral reef fishes develop red muscle tissue before other groups of fishes and larval reef fishes have high muscle tissue synthesis rates than other groups of fishes.

Chapter 5: Focal tropical coral reef fish exhibit high oxygen uptake rates throughout larval development, with concurrent changes in oxygen transport gene expression as fish develop.

Chapter 6: Oxygen uptake rates and muscle mitochondria volume densities decrease as reef fishes transition from the pelagic to reef habitats.

I address these hypotheses in my thesis within corresponding chapters, as well as via a literature review (**chapter 2**). **Chapter 4** was put on hold due to a global pandemic, but the concepts provide valuable links between chapters. **Chapter 7** is a general discussion that synthesises the findings from my chapters together within the context of my overarching hypothesis.

In **chapter 2** I review how marine teleost fishes develop organ and tissue systems that support swimming, outline the laboratory and field methodologies that measure swimming performance and their ecological relevance, and investigate how environmental stressors impact swimming during early life history. This chapter provides adequate context to justify measuring the physiological traits that are discussed in this thesis are relevant for reef fish ecology, and provides a foundation on which to develop hypotheses for **chapters 3-6**.

In **chapter 3**, I test hypothesis 1 and investigate how different biotic and abiotic factors influence swimming performance of marine teleost fish larvae. How does habitat association (reef, demersal, and pelagic), latitude (tropical versus temperate), and body morphology (i.e., size and shape) contribute to how swimming traits are expressed in larval marine fishes? Specifically, does the need to find a reef require higher swimming capabilities than simply remaining in the pelagic environment or finding a random benthic patch to settle on? To answer these questions, I take a comparative phylogenetic, data synthesis, and case-study approach to answer this question, and I aim to shed light onto the factors that may influence performance in fish larvae. The relationship between swimming and body size set up my experiments in **chapter 4**, investigating muscle tissue development during the larval phase of a coral reef fish.

Upon determining that body size plays an integral role in swimming performance among reef fishes in **chapter 3**, I use **chapter 4** to test hypothesis 2 by investigating how muscle tissue develops during embryonic and larval stages of a coral reef fish species. Specifically, is muscle tissue growth and development in this reef fish species different than other species of fishes? Using histochemistry techniques, I measured red muscle fiber growth and development over early ontogeny in an anemonefish. This further understanding of muscle tissue development in fishes living in a tropical ecosystem is important because high swimming performance during a short larval duration predictably relies on rapid muscle tissue development. High muscle development requires high oxygen uptake rates, thus setting the anatomical context for **chapter 5**.

In **chapter 5** of my thesis, I set out to answer hypothesis 3 by investigating oxygen uptake rates that support swimming performance and muscle tissue development, across the entire larval phase of a reef fish (cinnamon anemonefish, *Amphiprion melanopus*). I use swimming respirometry techniques to measure oxygen uptake during activity. I also sample larvae at specific timepoints in their larval stage to extract RNA to investigate changes in oxygen transport gene expression. This approach allows me to determine what the oxygen demands supporting swimming are over the entire larval period, whether there are distinct changes in oxygen uptake rates during development, and whether any associated changes in oxygen uptake are complimented by changes in genes associated with oxygen transport (e.g., haemoglobin) and oxygen delivery to tissues (myoglobin).

Lastly, my aim for **chapter 6** was to investigate the physiological mechanisms underpinning metamorphosis in coral reef fishes. Upon testing hypothesis 4, I was specifically interested in measuring changes in oxygen uptake rates and oxidative capacity of muscle tissues that may contribute to whole animal performance. Are these specific changes associated with shifting from pelagic to benthic habitats? Taking a field approach at Lizard Island (Australia), and using similar swimming respirometry techniques as in **chapter 5**, I swam settlement-stage larvae and settled juveniles of a common reef fish (Ambon damselfish, *Pomacentrus amboinensis*) and measured oxygen uptake rates during activity. Additionally, I collected tissue from swimming muscles (trunk and pectoral muscles) and stained the tissues to quantify muscle mitochondria volume density. This approach allowed me to determine whether there are changes in swimming performance as pelagic larvae settle onto their new benthic reef home, and whether this transition in lifestyle has any changes in oxygen demands during swimming and oxidative capacity of muscles.

In **chapter 7** I synthesized **chapters 2-6** to provide the whole story of how coral reef fishes develop into athletes, and I specifically highlight that understanding this question requires a multi-disciplinary approach to understand how, over development, changes at the molecular, cellular, and

tissue-level translate to whole-animal performance and, ultimately, ecosystem functions such as dispersal, connectivity, and recruitment.

In this thesis, I took a novel approach to understanding the physiology of larval fishes and the extreme changes in the physiology of tropical reef fishes that enables them to swim to high capacities. Taking a phylogenetic comparative approach to the role of habitat, latitude, and body size on patterns of swimming performance among fish larvae highlights the multitude of factors that may influence swimming performance. For the first time, oxygen demands that support activity over the entire larval stage have been measured, and cellular (i.e., changes in muscle mitochondria volume density) and molecular (i.e., haemoglobin and myoglobin gene expression) processes have been related to changes in whole animal performance. Addressing these knowledge gaps provides a better mechanistic understanding of the 'metabolic machinery' that supports high capacity swimming among coral reef fishes. From a wider perspective, coral reefs rely on reef fishes for many important ecological tasks to maintain coral health, and thus coral reefs rely on strong reef fish recruitment. Understanding the mechanism underpinning reef fish dispersal and recruitment will enable researchers to better predict how fish community dynamics may change in response to environmental stressors, such as climate change, as well as how reef fish population dynamics change to improve management of reefs (e.g., MPAs and fisheries). Therefore, investigating how energy requirements and structures responsible for swimming change during the larval phase is a critical step in furthering our understanding of coral reef fish life history, coral reef ecosystem dynamics, and conservation.

Chapter 2. Swimming performance of marine fish larvae: review of a universal trait under ecological and environmental pressure

2.1 Summary

The larval phase of marine teleost fishes is characterized by important morphological and physiological modifications. Many of these modifications improve the larvae's ability to swim, which satisfies a suite of crucial biological and ecological functions. Indeed, larval fish swimming performance has been considered a good proxy for overall condition, a predictor for growth and survival, and particularly helpful in assessing effects of natural and anthropogenic stress. Several methodologies have been developed to test larval fish swimming performance; however, measured swimming capabilities can strongly depend on the methodology utilised and developmental stage investigated. The aims were, therefore, to link the ontogenetic development of swimming performance in early life stages of marine fishes, particularly the anatomical and physiological processes around the fins, muscles, and gills, with both the experimental methodologies used and the environmental stressors tested. I conducted a literature search and found 156 research papers relevant to swimming performance of marine teleost fish larvae. I found swimming performance to be highly variable among species and driven by temperature. In a meta-analysis focusing on the impacts of environmental stress on larval swimming performance, I found that prey reduction had the greatest impact on swimming. Methods used to evaluate swimming should keep the ontogenetic stage a focus, as forced swimming experiments are unfit for larvae prior to flexion of the notochord. Overall, while the data are deficient in some areas, I am able to highlight where the field of larval fish swimming could be directed and provide insight into which methods are best used under certain ecological scenarios, environmental stressors, and developmental stages

Associated Publication

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Data availability

Data present in this chapter are available from the Research Data Repository (Tropical Data Hub) at James Cook University. DOI: https://doi.org/10.25903/ypyg-0p08

2.2 Introduction

With an estimated 17,000 known species, marine fishes are the most speciose group of vertebrates on the planet, and their importance for ecosystem function and global fisheries cannot be understated (Appeltans et al., 2012). Marine fishes act at all trophic levels (e.g., planktivores and herbivores, up to apex predators) and occur in a large range of ecosystems and latitudes (polar ice shelfs to tropical coral reefs). In order to maintain their populations, marine fishes rely on a continuous supply of larvae. At hatch, oviparous marine fishes are among the smallest free-living vertebrates (2-3mm total length) and generally do not possess many of the physical characteristics of juvenile and adult conspecifics (e.g., fins; Osse & Boogaart, 1999). Consequently, larvae are highly vulnerable to changes in environmental conditions, starvation, and predation, resulting in high mortality rates (~99.9% during the entire larval phase; Fuiman & Cowan, 2003; China & Holzman, 2014). Indeed, the larval phase of marine fishes is a period of developmental change characterised by morphological and physiological modifications. These changes enhance performance traits that subsequently support rapid development, improve chances of survival, and enable potential recruitment to the adult population. Since the early 1900s, fisheries biologists have attributed changes in marine fish population dynamics to larval fish survival; however, there is still a need for assessing the development of performance to better predict larval survival, in particular under changing environmental conditions (Houde, 2008). A highly relevant performance trait that encompasses important aspects of any marine fish's ontogeny, physiology, and ecology is swimming performance.

Swimming is a form of physical exercise powered by physiological and neuromuscular processes that enables fish to perform vital activities including foraging, avoiding predators, and undertaking daily and seasonal movements in their environment (Webb, 1984; Domenici & Blake, 1997; Hinch et al., 2005; Domenici & Kapoor, 2010). The appropriate anatomical structures for swimming and the physiological processes mediating them primarily develop during the larval phase and can be assessed by a number of metrics and techniques that have been developed to study the swimming performance of fish (Batty, 1984; Fuiman & Batty, 1997; Stobutzki & Bellwood, 1994). These methods have been used to investigate effects of different intrinsic and extrinsic factors on swimming performance, and to assess their implications for ecologically-relevant processes. For example, swimming is directly impacted by abiotic factors, such as temperature (Batty & Blaxter, 1992; Green & Fisher, 2004; Hunt von Herbing, 2002; Moyano et al., 2016; Wieser & Kaufmann, 1998) and carbon dioxide (CO_2) levels (Pimentel et al., 2014; Pimentel et al., 2016; Silva et al., 2016), and biotic factors such as prey availability (Illing et al., 2018). Knowledge as to how larvae respond to such changes is not only key for their individual survival, but also

for predicting important events, such as dispersal or recruitment to adult stocks (Hufnagl & Peck, 2011; Huebert & Peck, 2014; Huebert et al., 2018).

Considering the increasing anthropogenic pressure on marine fishes, the use of larval fish swimming as a universal performance trait holds great potential for both a better mechanistic understanding of organismal performance and for assessing consequences on larger-scale levels, such as predicting dispersal and recruitment. However, there is a need to integrate the ontogenetic development of swimming performance, the metrics by which swimming is measured, and how changes in the environment will impact swimming at different stages of larval development. The aims of this chapter are, therefore, to i) integrate morphological and physiological perspectives with the development of swimming performance, ii) evaluate the ecological relevance of commonly used methodologies to measure swimming, and iii) assess how (anthropogenic) environmental stressors affect swimming performance of marine fish larvae. Additionally, throughout the chapter, I attempt to disentangle the effects of latitudinal background, (i.e., the climatic region) and taxonomic origin on development and environmental impacts on swimming performance.

2.2.1 Methods

I conducted a systematic literature search to find all relevant studies (ISI Web of Knowledge, Clarivate Analytics, Core collection search on 19.02.2019 using the term: ((swim* OR sust* OR prolong* OR burst* OR cruis* OR routin* OR Ucrit OR endur*) AND (early life stage* OR larv*) AND (marin* OR sea* OR brack*) AND (fish* OR teleost*))). This search resulted in 1,938 papers that were then checked for suitability, subsequently providing 156 studies relevant to my aims. I proceeded to extract data from the aforementioned 156 studies using WebPlotDigitizer (Version 4.2, Ankit Rohatgi). I grouped the retrieved data into climatic regions and taxonomic orders based on information provided in Fishbase (Froese & Pauly, editors, 2017). Average daily growth and pelagic larval duration information incorporated into Figure 3 was obtained from Fishbase (Froese & Pauly, editors, 2017). Furthermore, I compared the swimming performance (expressed as Reynold's Number; *Re*) of marine larvae across different ontogenetic stages and climatic regions. Reynold's number (*Re*), the dimensionless unit describing the ratio of inertial to viscous forces, was calculated for each larva as

$$Re = U_{crit} \times L \times \nu^{-1}$$

where critical swimming speed (U_{crit}) and body length (L) are specific for each larva and ν is the kinematic viscosity of seawater at different temperatures (Webb & Weihs, 1986).

All statistical analyses were conducted in R (R Development Core Team, 2013), e.g., model selection, diagnostics, and post-hoc tests ('lme', 'emmeans', and MuMin). Studies assessing the effects of environmental stress on swimming performance were grouped into categories of ocean acidification (OA), ocean warming (OW), ocean acidification and warming (OAW), prey reduction, and toxicants. I did not include studies that expressed swimming performance as percentage active vs. inactive, as I compared all studies using an absolute value for swimming performance (e.g., cm s^{-1}). I conducted a multivariate meta-analysis to determine the effect of the respective environmental stressors on swimming performance of marine fish larvae, using R and the package "metafor" (Viechtbauer, 2010).

2.3 Ontogenetic Development and Hydrodynamic Limitations

The early life history of most marine fishes is characterised by a larval phase, which is spent in the open ocean and often referred to as the pelagic larval duration (PLD; Cowen & Sponaugle, 2009). This period can be further divided into a pre-competent and a competent phase (Jackson & Strathmann, 1981). These two phases are essentially separated by the ability of a fish larva to overcome hydrodynamic processes affecting its dispersal (e.g., ocean currents). This transition is influenced by extrinsic (i.e., effects from the abiotic and biotic environment) and intrinsic (i.e., development of key swimming structures and the physiological processes mediating them) factors.

At hatch, most marine teleost larvae are not fully developed and lack many important structures crucial for swimming: i) the caudal fin, the most important structure for horizontal swimming, has not been formed yet, ii) gills are not present (cutaneous oxygen uptake is sufficient), and iii) muscles are undifferentiated (Figure 2.1; Blaxter, 1988; Müller & Videler, 1996). This simple form, known as the pre-flexion stage, limits swimming capabilities regardless of climatic region or taxonomic group (Figure 2.2b and c); however, it does allow larvae to change their horizontal displacement by swimming vertically in the water column (Figure 2.1; Voesenek et al., 2018). Without a properly formed caudal fin, most larvae use high amplitude body undulations to swim (e.g., such as anguilliform swimming representative of many herring species), which can be energetically expensive (Figure 2.1; Müller et al., 2008; Yavno & Holzman, 2018). Indeed, at this developmental stage swimming can use up to 80% of a larval fish's energy budget (e.g., Atlantic cod; *Gadus morhua*; Ruzicka & Gallager, 2006). A crucial developmental milestone is the flexion of the notochord, a rudimentary support structure for the vertebral column, which forms the caudal fin (Figure 2.1). This stage, known as post-flexion, marks an increase in swimming competency for marine fish larvae and enables them to effectively overcome the limitations of their hydrodynamic environment. Notochord flexion generally occurs in conjunction with gill and muscle development to facilitate oxygen delivery to tissues and increase locomotor output, respectively.

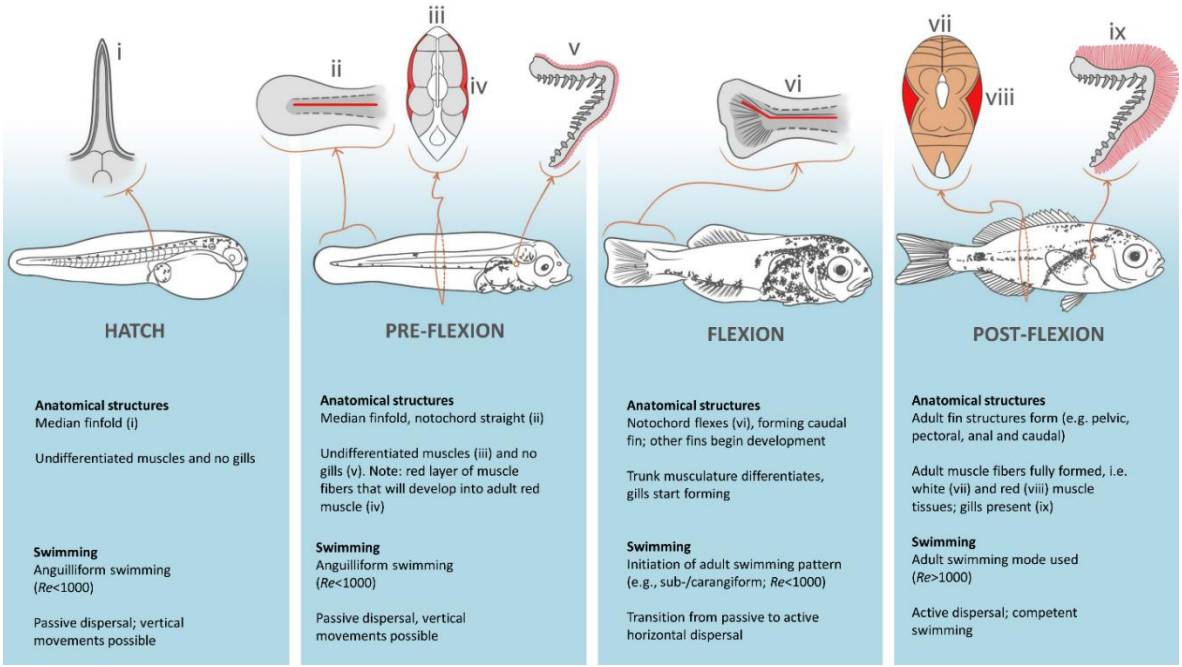

Figure 2.1 Generalized overview of how swimming capabilities develop throughout early ontogeny in marine fishes. Each ontogenetic stage is described in terms of the anatomical structures that form (e.g., muscles, gills, and fins, highlighted by cross-sectional or top views), and how they influence swimming performance (e.g., Reynold's Number (*Re*), swimming mode, and vertical/horizontal swimming capabilities).

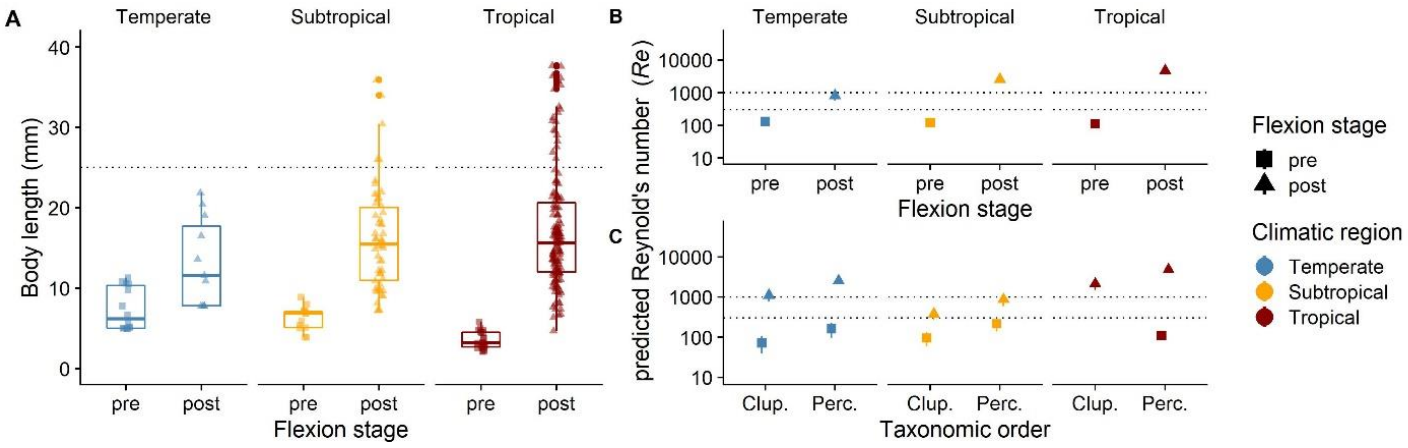

Figure 2.2 A) Body lengths of pre- and post-flexion stages of marine fish larvae from three climatic regions. Marine fish larvae develop faster in warmer climatic regions, and undergo the flexion of the notochord, a developmental milestone for improving swimming performance, at smaller body sizes. Original data were retrieved from a systematic literature search (see methods). I used body length, the inverse of the kinematic viscosity of seawater, and critical swimming performance (U_{crit}) data to calculate the Reynold's number (*Re*), a dimensionless unit describing the ratio of inertial to viscous forces. Limiting the body length data set to <25 mm (dotted line) did not provide better model results. B) Modelled means (± 95 % CI) of the Reynold's number that marine fish larvae experience in waters of different climatic regions, separated by pre-and post-flexion stages (total body length range 2-38 mm). C) Modelled means (± 95 % CI) of the Reynold's number that pre- and post-flexion stages of marine Clupeiform and Perciform fish larvae experience across climatic regions. Dotted lines indicate the transition area from the viscous to the inertial zone, where competent swimming is achieved (*Re* = 300 – 1000; note logged y-axis scales in panels B+C). See tables 2.1 and 2.2 for details on statistical models.

Table 2.1 Summary table from emmeans function (emmeans package), comparing *Re* between regions (temperate, subtropical, tropical), using all pre- and post-flexion stages, as per Fig 2.2a. Results are given on the log-scale. Tukey method was used for p-value adjustments.

Model: lm(log(Re)~Region

Region	Emmean	SE	df	lower.CL	upper.CL
Temperate	5.78	0.196	250	5.40	6.17
Subtropical	6.32	0.150	250	6.03	6.62
Tropical	6.58	0.114	250	6.36	6.80

Contrast	Estimate	SE	df	t.ratio	p.value
Temperate-Subtropical	0.541	0.247	250	2.191	0.0746
Subtropical-Tropical	-0.256	0.188	250	-1.362	0.3623
Temperate-Tropical	-0.797	0.226	250	-3.522	0.0015

Table 2.2 Summary table from emmeans function (emmeans package), comparing *Re* between regions and stages, as per Fig. 2.2b. Results are given on the log-scale. Tukey method was used for p-value adjustments.

Model: lm(log(Re)~Region*Stage

Region	Stage	Emmean	SE	df	lower.CL	upper.CL
Temperate	Preflexion	4.87	0.2597	250	4.36	5.38
Subtropical	Preflexion	4.80	0.2695	250	4.27	5.34
Tropical	Preflexion	4.70	0.2121	250	4.29	5.12
Temperate	Postflexion	6.69	0.2930	250	6.12	7.27
Subtropical	Postflexion	7.84	0.1323	250	7.58	8.10
Tropical	Postflexion	8.46	0.0813	250	8.30	8.62

Contrast	Stage	Estimate	SE	Df	t.ratio	p.value
Temperate-Subtropical	Preflexion	-0.0671	0.374	250	-0.179	1.0000
Subtropical-Tropical	Preflexion	0.1009	0.343	250	0.294	0.9997
Temperate-Tropical	Preflexion	0.1680	0.335	250	0.501	0.9961
Temperate-Subtropical	Postflexion	1.1483	0.321	250	3.572	0.0056
Subtropical-Tropical	Postflexion	-0.6138	0.155	250	-3.954	0.0014
Temperate-Tropical	Postflexion	-1.7621	0.304	250	-5.795	<.0001

Cutaneous respiration becomes inefficient as larvae increase in size, which is based on species-specific growth rates and species-specific oxygen requirements per unit mass (QO_2; Blaxter, 1988), and thus gills are crucial to provide sufficient oxygen to all aerobically driven tasks. Generally, upon gill formation, fish larvae possess high mass-specific oxygen uptake rates compared to adult conspecifics, which is associated with high metabolic rates to support rapid tissue development (Post & Lee, 1996; Killen et al., 2007; Peck & Moyano, 2016). However, the body of literature on oxygen consumption rates of marine teleost larvae is sparse, mainly due to the logistics of measuring oxygen consumption of small sizes of marine larvae (Peck & Moyano, 2016). In fact, few studies have investigated the oxygen consumption rates of swimming larvae, and >95% of the existing studies have tested only temperate fish larvae (Peck & Moyano, 2016). Gill development also occurs in concert with muscle tissue differentiation (El-Fiky et al., 1987). As muscle tissue separates and forms red and white muscle types (i.e., differentiation) and muscle fiber abundance increases (i.e., hyperplasia), fish start utilizing a swimming mode analogous to the adult stage (e.g., sub-carangiform or carangiform, see references in Müller, 2008; Figure 2.1). The thin layer of muscle responsible for cutaneous respiration will develop into red muscle tissue characteristic of adult stages that powers sustained swimming behaviours (Figure 2.1; Rombough, 1988). Taken together, these anatomical and physiological modifications help fish larvae to become competent swimmers and escape their viscous environment.

The viscosity of water has a proportionally stronger effect on smaller organisms compared to larger organisms and therefore hinders effective swimming of many marine fish larvae. A dimensionless metric, called the Reynold's number (*Re*), describes the ratio of the viscous forces of water against an animal and the inertial forces of an animal moving through water (Taylor, 1951; Fuiman & Batty, 1997). A low *Re* (<300) indicates that the animal's swimming capabilities are not strong enough to overcome the viscosity of the water (viscous zone; pre-competent phase); whereas, high *Re* (>1000) is attributed to inertial forces of the swimming animal being greater than the resistive forces of the water (inertial zone; competent phase; Figure 2.1; Ngo & McHenry 2014; Peck & Moyano 2016). Several factors impact the *Re*, including size, swimming speed, and water viscosity, all of which are highly temperature-dependent (Figure 2.2 and 2.3; Hunt von Herbing, 2002). Thus, for example, the higher *Re* of fish larvae inhabiting tropical latitudes compared to fishes of temperate environments is partially attributed to warmer water temperatures (Figure 2.2b). Regardless of climatic region, investing energy into anatomical structures designed for swimming mitigates the challenges of such a viscous environment, which can mean "the difference between acquiring energy and being acquired energy" (Goolish, 1991).

To date, much of what we know about the development of marine fishes relates to those that inhabit temperate environments. Marine fishes inhabiting tropical and polar (stenothermal) habitats may

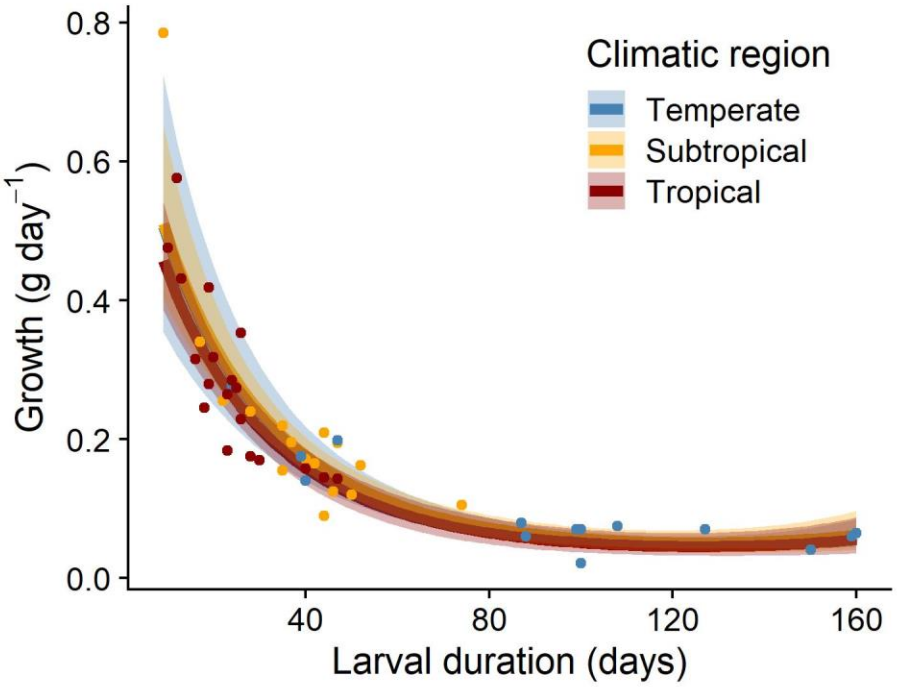

Figure 2.3 Average growth (g body mass day^{-1}) of marine fish larvae from different climatic regions and their larval duration (days before metamorphosis). Data were extracted from Fish Base, and each data point represents an individual species. An exponential curve was fit through the data (log (growth)~larval duration + climatic region + I(larval duration^2; df = 44, $p < 0.001$, $R^2 = 0.85$). Bands represent 95% CI. For details on statistical output, refer to Table 2.3.

Table 2.3 Summary table from emmeans function (emmeans package), comparing average daily growth and larval duration between climatic regions (temperate, subtropical, tropical). Results are given on the response scale but tests were performed on the log-scale. Tukey method was used for p-value adjustments.

Model: lm(log(Growth))~Larval duration+Region+I(〖Larval duration〗^2)

Region	Response	SE	Df	lower.CL	upper.CL
Temperate	0.136	0.0140	44	0.111	0.167
Subtropical	0.138	0.0103	44	0.118	0.160
Tropical	0.123	0.0119	44	0.101	0.150

Contrast	Ratio	SE	df	t.ratio	p.value
Temperate-Subtropical	1.01	0.133	44	0.082	0.9963
Subtropical-Tropical	1.12	0.114	44	1.104	0.5168
Temperate-Tropical	1.11	0.166	44	0.674	0.7798

have some differences in their developmental patterns when compared to their temperate water counterparts. However, in contrast, most available data on swimming performance is from tropical perciform fishes, likely due to the large number of species occurring in the tropics, and the high number of species encompassed by Perciformes (Leis, 2007; Leis et al., 2013b). Regardless, at any given size, tropical marine fish larvae, particularly those that hatch from demersal eggs (e.g., anemonefishes), are better developed (e.g., in terms of fin and sensory systems), and have higher growth rates than their temperate counterparts, such as herrings or cods (Mccormick & Leis, 2002; Leis, 2007; Leis et al. 2013b). Flexion of the notochord appears to occur at a smaller body size for tropical compared to temperate larvae (Figure 2.2a). After extracting data from the original literature, I predicted *Re* with an overall linear model, and found no differences in *Re* were observed in pre-flexion life stages across climatic regions ($p>0.05$), whereas all post-flexion life stages differed significantly from another ($p<0.01$) (Figure 2.2; see Table 2.1 and 2.2 for details on statistical output). Many factors, such as life history traits (e.g., developmental rates), morphology, and evolutionary history are hypothesized to contribute to this difference in marine fish ontogeny and swimming performance; however, temperature is widely regarded as a leading factor (Leis et al., 2013b).

To investigate whether taxonomy or latitude has the greater impact on swimming performance (represented by *Re*), I compared two taxonomic groups of fishes, Order Perciformes and Order Clupeiformes. These are the only two orders with sufficient swimming data available across a wide latitudinal range (temperate, subtropical and tropical climate regions) and for most developmental stages (Figure 2c). Perciformes are more speciose in the tropics, and are considered better swimmers than Clupeiformes, which are more abundant in temperate regions. I analyzed a subset of the data (only Clupeiform and Perciform fishes) and added taxonomic order as a fixed factor to the model (log(Re) ~ Order + Stage * Region; df=221, $p < 0.001$, $R^2 = 0.68$). I used Tukey post-hoc tests for pairwise comparisons between the groups, and found no significant differences in *Re* between taxa when compared within the respective developmental stage (pre- and post-flexion; $p >0.05$; Figure 2c). While this is based on comparing two groups with limited data, it may suggest that temperature is a greater driver of performance than taxonomic group alone, as larvae of marine fish species living in warmer climatic regions posses a higher capacity for swimming, regardless of taxonomic order.

2.4 Methodologies and Ecological Relevance

The most widely utilised methodology to measure larval fish swimming performance is routine swimming, which is evaluated by measuring the swimming speed of undisturbed larvae or quantifying behaviour as ‘percent active versus inactive’ (e.g., Fisher & Bellwood, 2003). Routine swimming tests provide valuable information as to how larvae naturally interact with their environment under both natural

(e.g., swimming speed as larvae develop; Ryland, 1963; Fuiman et al., 1999; Fisher & Bellwood, 2003; Arndt et al., 2016; Garrido et al., 2016; Højgaard et al., 2018) and modified environmental conditions in laboratory settings including stressors, such as prey reduction (Chick & Van Den Avyle, 2000), ocean warming (Moyano et al., 2016) and ocean acidification (Rossi et al., 2015), or exposure to toxins (Benítez-Santana et al., 2014). Some studies, mainly on coral reefs, have quantified routine swimming performance of larval fishes in the field, whereby divers released captured tropical coral reef fish larvae back onto the reef and followed them *in situ* to quantify their swimming speed, depth, orientation, and interactions with other fishes (Leis & Carson-Ewart, 1999, 2001, 2002; Leis et al., 1996; Leis & Carson-Ewart, 1997, 1998). Measuring routine swimming in the field provides i) valuable information regarding natural swimming speeds of larvae as they swim toward the reef to settle (Leis & Carson-Ewart, 1997, 1998, 1999, 2001, 2002) and ii) allows for comparisons to be made between *in situ* swimming speeds and other laboratory measures (Fisher et al., 2005; Fisher & Leis, 2010). Unfortunately, the effect that the diver has on swimming performance of the observed larvae is not known (Leis, 2006). Yet, routine swimming, especially *in situ* swimming speeds, would provide highly valuable information for dispersal models, as these are minimum speeds larvae swim at under natural conditions.

An endurance swimming test involves swimming a fish at a fixed water velocity until the fish fatigues, and is generally repeated using different flow intensities (e.g., high-water flow, such as U_{crit}, see below, down to routine swimming speeds of 1 body length (BL s^{-1})). This helps create a fatigue curve to determine i) how long fish can swim at various ecologically-relevant speeds (e.g., different oceanic currents or tides; see Peake et al., 1997) and ii) when fish transition between different modes of swimming (e.g., sustained, prolonged, and burst; see Beamish, 1978). For many temperate fish larvae, competent swimming is not possible until much later in ontogeny, generally when fish approach the juvenile phase. However, it may be an important metric for tropical fish larvae, due to their remarkable swimming capabilities early in their development (for examples of swimming speeds and notes on ecological relevance, see Stobutzki & Bellwood 1994; Leis et al., 1996; Jones et al., 1999; Fisher et al., 2005; Fisher & Leis, 2010). For example, when provided a routine feeding regime, cinnamon anemonefish (*Amphiprion melanopus*) larvae (5.5-7.6 mm total length (TL)) are capable of swimming at 7 cm s^{-1} for 50 h (maximum of 120 h) and covering 12 km (maximum of 30 km; Fisher & Bellwood, 2001). While it has not been evaluated to date, information on energy requirements for sustained swimming performance at ecologically-relevant flow velocities would be valuable in determining cost of transport (COT; amount of energy required to move a unit distance) during dispersal, settlement, and recruitment processes. Integrating oxygen uptake, a proxy for energetic costs, into physiologically-coupled biophysical models will help improve predictive power and help assess potentially increased

costs under changing environmental conditions. This knowledge gap has set the stage for **chapters 5 and 6** of my thesis.

The most widely used metric to measure swimming performance of adult fishes, and also common among larval fishes, is the critical swimming test (U_{crit}; Brett, 1964). The U_{crit} test (Brett, 1964) is a stepped-velocity test, whereby water velocity increases by reliable increments (generally 1 BL s^{-1}) after a fixed time interval until the fish fatigues. The U_{crit} test has been used to evaluate how swimming performance develops throughout the larval phase of many marine fishes (Faria et al., 2009; Faria & Goṇalves, 2010; Leis et al., 2006, 2012), compare swimming competencies of different species/families (e.g., Fisher et al., 2005), and evaluate how environmental changes impact swimming performance (Green & Fisher, 2004; Guan et al., 2008; Koumoundouros et al., 2009; Moyano et al., 2016; Munday et al., 2009). However, the test has been criticized for a few reasons: i) altering the time and speed intervals may drastically change U_{crit} (Farlinger & Beamish, 1977; Downie & Kieffer, 2017, but see Hogan & Mora, 2005), ii) swimming the fish until fatigue involves anaerobic metabolism (i.e., the U_{crit} test is not an obligatory aerobic measure per se), and iii) fish do not generally swim at U_{crit} for time periods relevant to daily/seasonal movements. As such, U_{crit} should be used with caution when modelling dispersal, and thus, endurance swimming and routine swimming should be considered as more accurate metrics (see reviews by Plaut, 2001; Fisher & Leis, 2010; Majoris et al., 2019). I recommend the use of U_{crit} when comparing the magnitude by which an environmental stressor impacts swimming performance. A valuable application for U_{crit} would be to measure oxygen uptake at each swimming speed so that a COT/fatigue graph over a wide range of water flow velocities could be created to determine swimming efficiency, a critical knowledge gap addressed in **chapter 6** of my thesis. Also, aerobic scope (an animal's aerobic energy budget) can be calculated from swimming fish, which may be a more accurate method than static respirometry (Rummer et al., 2016a). While this would increase the experimental time (i.e., 10-20 min per step interval, versus the generally used 2-5 min per interval), the data would be invaluable in determining, for example, the costs for swimming at different speeds, whether the oxygen budget changes over ontogeny, and the impact of environmental stress on oxygen uptake rates during activity. These techniques are used in **chapters 5 and 6** of my thesis to better understand the aerobic scope of reef fish larvae.

Burst swimming is associated with larvae capturing prey and avoiding predators (e.g., Batty et al., 1993) and produces the fastest swimming speeds. Burst swimming is generally captured using a high-speed camera and software to quantify these fast movements (see review by Domenici & Blake, 1997). Burst swimming has been used to understand effects of varying prey densities (Faria & Goṇalves, 2010; Faria et al., 2011) or chemicals (e.g., (Alvarez et al., 2006; Johansen et al., 2017). Several studies have

also investigated how burst swimming develops throughout the larval phase and across taxa (notable examples include (Masuda et al., 2002; Masuda, 2006; Benítez-Santana et al., 2007; Chesney, 2008; Olivier et al., 2013). The implications for burst performance have been incorporated into individual-based models (IBMs) associated with growth and survival, across many developmental stages (Peck & Hufnagl, 2012), and the methods are of particular interest in determining how fish larvae may be impacted by environmental stress (e.g., Allan et al., 2015).

2.5 Environmental Impacts

2.5.1. Ocean Acidification (OA)

Ocean acidification (OA) is defined as the decline in ocean water pH due to the absorption of atmospheric CO_2 (Lopes et al., 2016). Adult and juvenile teleosts may be more resistant to OA, as they are quite capable of efficient acid-base regulation (Munday et al., 2009; Bignami et al., 2014; Rummer & Munday, 2017); however, such regulation is energetically expensive and may have ripple effects on other physiological processes and overall performance and behaviour (Munday et al., 2009; Leis, 2018). Teleost larvae are believed to lack many physiological mechanisms that allow more developed life-stages to tolerate environmental conditions, including OA, and consequently, it is hypothesized that early life stages of marine fishes are highly susceptible to the effects of acidification. (Bignami et al., 2013; Leis, 2018). While several studies have shown negative, sub-lethal, impacts on larval fish predator avoidance (Dixson et al., 2010), vision (Chung et al., 2014), lateralization (Domenici et al., 2012), hearing (Simpson et al., 2011), learning (Ferrari et al., 2012), and activity rates (Munday et al., 2010), OA does not seem to impact swimming performance, regardless of climatic region or swimming methodology (Figure 2.4). Tropical fishes, such as the common dolphinfish (*Mahi mahi),* possess high metabolic rates, which may allow for increased ability to regulate pH (Bignami et al., 2013, 2014), and there is evidence that young *Mahi mahi* supress metabolic rates under OA conditions, creating a trade-off with a decreased growth rate (Pimentel et al., 2014). The larvae of barramundi (*Lates calcarifer*), a tropical, euryhaline fish, slightly decrease routine swimming activity in response to exposure to OA, which may have impacts on timing of recruitment and growth rates (Rossi et al., 2015). Similarly, larvae of temperate fish, like herring, do not show a change in routine swimming capabilities (Figure 2.4; Maneja et al., 2015).

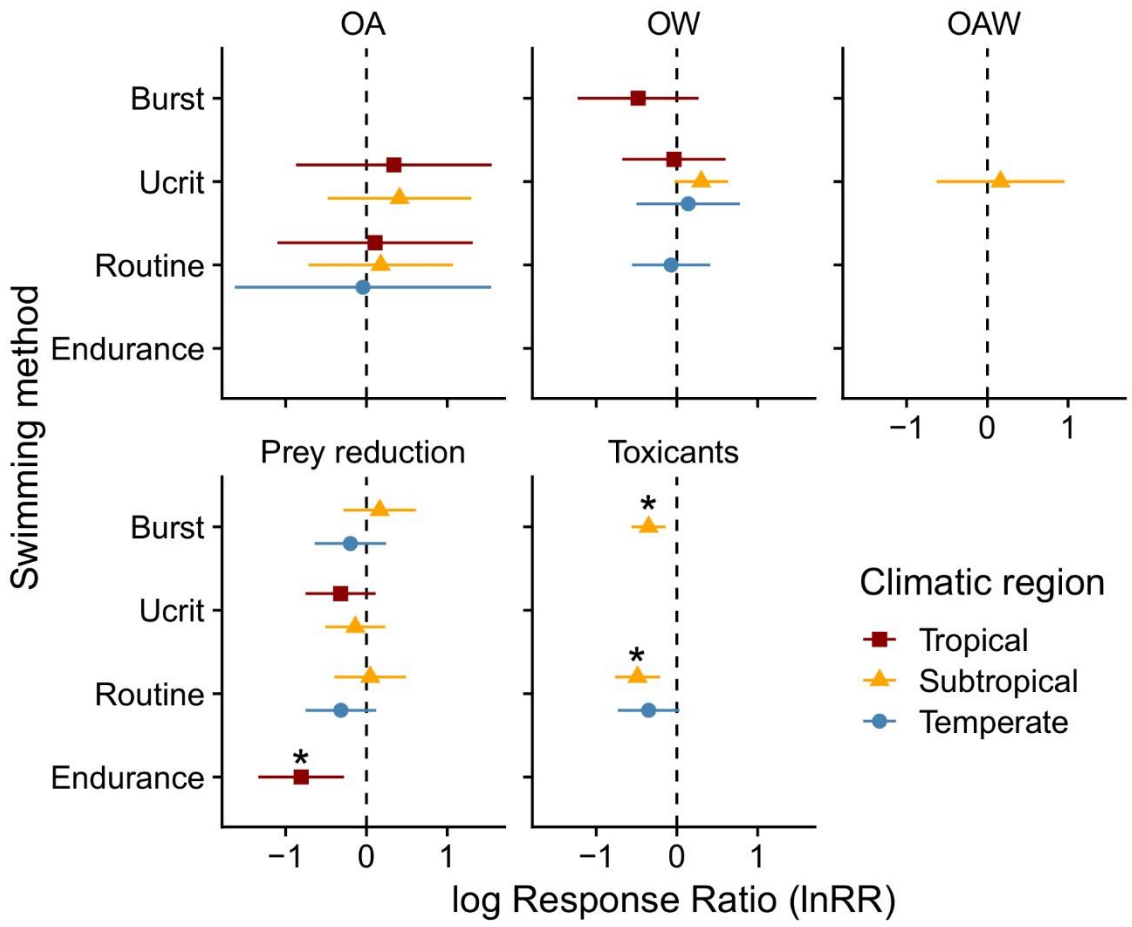

Figure 2.4 Mean effect sizes (lnRR) of environmental factors on swimming performance measures of marine fish larvae. Studies investigating the effects of ocean acidification (OA), ocean warming (OW), the combination of ocean acidification and warming (OAW), prey reduction, and toxicants were retrieved by a systematic literature search in the Web of Science (n = 27). The original data were extracted, converted to swimming speeds in cm s^{-1}, and compared using multivariate meta-analyses (see methods for further details). Symbols indicate predicted mean effect sizes (± 95% CI), and are shape-and color-coded by climatic regions. Significant effects ($p < 0.05$) were observed where the confidence bands did not span zero (marked with asterisks).

Table 2.4 Model summaries for the impact of environmental stressors (ocean acidification, ocean warmong, ocean acidification and warming, prey reduction and toxicants) on swimming performance of larvae at different regions (temperate, tropical, subtropical) and methodologies (U_{crit}, routine, endurance or burst)

Environmental Stressor	Method/Region	Estimate	SE	z	P	Lower CI	Upper CI
OA	Routine	0.18	0.46	0.39	0.69	-0.72	1.01
	U_{crit}	0.41	0.45	0.09	0.37	-0.48	1.3
	Temperate	-0.22	0.93	-0.24	0.81	-2.04	1.6
	Tropical	0.07	0.57	-0.12	0.9	-1.19	1.05
OW	Burst	-0.14	0.53	-0.27	0.79	-1.18	0.9
	Routine	0.09	0.44	0.2	0.84	-0.78	0.96
	U_{crit}	0.3	0.17	1.78	0.08	-0.031	0.63
	Temperate	-0.16	0.37	-0.44	0.66	-0.88	0.56
	Tropical	-0.34	0.37	-0.92	0.36	-1.06	0.38
	U_{crit}	0.16	0.4	0.41	0.69	-0.63	0.96
PR	Burst	0.16	0.23	0.72	0.47	-0.29	0.61
	Endurance	-0.63	0.31	-2.01	0.045	-1.24	-0.015
	Routine	0.047	0.23	0.21	0.84	-0.39	0.49
	U_{crit}	-0.14	0.19	-0.73	0.47	-0.51	0.23
	Temperate	-0.36	0.26	-1.39	0.16	-0.88	0.15
	Tropical	-0.18	0.15	-1.19	0.23	-0.48	0.12
Tox	Burst	-0.35	0.11	-3.23	0.001	-0.56	-0.14
	Routine	-0.49	0.14	-3.43	<0.001	-0.77	-0.21
	Temperate	0.14	0.24	0.57	<0.001	-0.34	0.61

OA = ocean acidification, OW= ocean warming, OAW= ocean warming an acidification, PR=prey reduction, Tox = toxicants

Based on the limited data, it is possible that teleosts are capable of acid-base regulation to some capacity early in development (Lopes et al., 2016). The reallocation of resources and energy to acid-base regulation could explain the lack of significant change in swimming performance during exposure to OA conditions, and the negative impacts OA has on larval growth and development (Silva et al., 2016). Species inhabiting habitats with high natural fluctuation in pH, such as those on coral reefs, may be more resilient and able to keep acid-base balance under hypercapnic conditions (Michaelidis et al., 2007; Munday et al., 2009). While speculative, the exact mechanism underpinning the lack of impact OA has on swimming at such an early ontogenetic stage is unknown, and it cannot be understated that there is generally a lack of available studies on the subject.

2.5.2. Ocean Warming (OW)

Temperature governs all biochemical processes and basic physiological functions. Thus, ectotherms, such as fishes, strongly depend on temperature, as it controls metabolism and regulates factors relevant for fast growth and developing structures required for swimming (Rummer & Munday, 2017). However, these processes occur within an optimal temperature window, and any direction, warmer or colder, outside this window causes performance to deteriorate (Rombough, 1997). Ocean warming (OW) is becoming an ever-present threat to our ecosystems, and ectotherms operating outside of their optimal thermal windows may face the risk of being unable to sustain their metabolic rates (Moyano et al., 2016). Teleost larvae are especially at risk, as they are believed to be incapable of physiologically multi-tasking between high growth rates, swimming, basic maintenance costs, and responding to stress (Killen et al., 2007). As a result, understanding how OW will impact swimming performance is crucial.

Generally, swimming speed increases with temperature due to a reduced viscosity of the water and an increase in the activity of swimming muscles (Batty et al., 1991, 1993). In concert with this, herring (*Clupea harengus*) larvae reared at warmer temperatures grew faster, achieved developmental milestones sooner, and performed better than conspecifics reared at lower temperatures (Moyano et al., 2016). Atlantic cod (*Gadus morhua*, 5 dph; 5mm TL) swim circa 70% faster at 10 °C than at 0 °C (Hunt von Herbing & Keating, 2003). Cinnamon anemonefish (*Amphiprion melanopus*) reared at 25 °C swam to a lesser capacity than those reared and swum at 28 °C (U_{crit} protocol; Green & Fisher, 2004). However, (Koumoundouros et al., 2009) found that the optimum swimming temperature for gilthead seabream (*Sparus aurata*) was 25°C, as they found a decrease in U_{crit} at 28 °C. Additionally, the escape response of Ward's damselfish (*Pomacentrus wardi*) was reduced at elevated temperatures due to a decrease in muscle power at high temperatures (Allan et al., 2015). These two studies (Koumoundouros et al., 2009; Allan et al., 2015) show how operating outside of a fish's optimal thermal window many hinder performance and subsequently survival.

Across all climatic regions and methods, I found no overall significant change in swimming performance for marine teleost larvae in response to OW (Figure 2.4). This may be attributed to several factors, such as i) high levels of individual variation (Moyano et al., 2016, Hunt von Herbing & Keating, 2003), ii) narrow temperature ranges tested (Klumb et al., 2003), iii) pooling together pre- and post-flexion larvae for analyses (Hunt von Herbing & Keating, 2003; Moyano et al., 2016), and iv) testing species with wide thermal windows (e.g., subtropical species) that are often not impacted by large increases in temperature (Koumoundouros et al., 2009). At the smallest sizes (i.e., generally pre-flexion), larvae are not fully developed (i.e., lacking proper muscles and fin structures; Figure 2.1), and thus swim poorly, regardless of experimental temperature (Hunt von Herbing & Keating, 2003; Moyano et al., 2016; See Figure 2.2b and c). As larvae increase in size and develop further, temperature impacts swimming more, than the viscosity of the water (Hunt Von Herbing & Keating, 2003). Temperate and subtropical larvae with wider thermal windows tend to increase their critical swimming performance, likely benefiting from faster growth and development at elevated temperatures (Figure 2.4).

2.5.3. Ocean Acidification and Ocean Warming (OAW)

Generally, most laboratory experiments test swimming performance of larvae against OA and OW scenarios separately. However, these processes are co-occurring, with the nature of the stressor interactions still being unclear (Baumann, 2019; Laubenstein et al., 2018, 2019). Ocean acidification effects are often hypothesized to amplify OW effects, either additively or synergistically (Watson et al., 2018; Cominassi et al., 2019). Co-occurring warming and acidification effects potentially increase metabolic demands and may divert additional energy from growth to acid-base regulation (Bignami et al., 2017). However, evidence suggests that, in trials with concerted warming and elevated CO_2, elevated temperature has the greater impact on swimming performance (Figure 2.4; Bignami et al., 2017; Watson et al., 2018; Cominassi et al., 2019). I am only aware, to date, of three studies combining OA and OW to investigate the effects on swimming performance (Bignami et al., 2017; Cominassi et al., 2019; Watson et al., 2018). Thus, it is difficult to draw final conclusions using such few studies, geographic regions represented, and methodologies used (Figure 2. 4). In the three present studies on subtropical fish larvae, OAW did not affect critical swimming performance (Figure 2. 4). However, multi-stressor experiments are the most promising approach to disentangle effects of co-occurring and interacting climate change factors. Future studies, with a focus on a wider taxonomic range, methods, developmental stages, and geographic regions will provide stronger evidence for how these combined stressors may impact swimming performance.

2.5.4. Prey Reduction

The two main drivers of larval fish mortality are starvation and predation, and since the early 1900s, many experimental and field studies have shown slow-growing and low-performing fish to be more vulnerable to predation (e.g., Hjort, 1914; Takasuka et al., 2004). Therefore, fast growing fish larvae that manage to optimize their feeding behaviour are positively selected for, rendering a flexible search behaviour as a key trait for survival in prey scarce environments. When prey is not limiting, larvae decrease swimming speed to save energy, and consequently decrease their encounter rate with predators (Chick & Van Den Avyle, 2000; Mahjoub et al., 2011). When prey becomes limiting, however, some fishes increase their search behaviour until they reach a "point of no return" (Hempel & Blaxter, 1963). Results from my meta-analysis on the effects of prey reduction on swimming performance indicate that most marine fish larvae show an overall trend toward a decrease in performance after periods of food shortage. Still, only endurance swimming was significantly negatively affected (Figure 2.4). Several factors contribute to the variability in the findings. First, some studies investigated pre-flexion larvae that had not fully developed their swimming capacity (Faria et al., 2011). Second, the period that larvae fasted or were starved differed between studies (Skajaa & Browman, 2007; Faria et al., 2010; Faria et al., 2011; Mahjoub et al., 2011). Third, specifically for burst swimming, muscle glycogen stores may be maintained during periods of starvation (Floyd & Anderson, 2010). The maintenance of short-term swimming performance in prey-reduced environments may be a strategy to conserve energy and prioritize behaviours that help capture food when it becomes available again, and escape predators (Skajaa & Browman, 2007; Faria et al., 2010; Faria et al., 2011)

Prey availability studies are the only larval fish swimming studies to use endurance tests as a metric for measuring swimming performance (Fisher & Bellwood, 2001; Leis & Clark, 2004; Faria et al., 2011). Feeding the larvae during endurance swimming experiments provides a more accurate measure as to how far a fish can travel, as they 'feed on the run', before switching to the juvenile and adult life styles and habitats (Leis & Clark, 2004). Across all studies, larval fish, where food was withheld, swam shorter distances than larvae that were fed (Figure 2.4; Fisher & Bellwood, 2001; Leis & Clark 2004; Faria et al., 2011), and it has been suggested that endurance swimming is limited by energy reserves availability (Faria et al., 2011). Therefore, unfed larvae undergoing endurance experiments are an indicator of how far energy stores can take larvae (Stobutzki, 1997). Regardless of climatic region, endurance swimming experiments, where fish are fed and their oxygen uptake quantified, will be an invaluable metric to evaluate travel distances of larvae and related costs.

2.5.5. Toxicants

The two main toxicants that have been used to evaluate the effects of chemicals on swimming performance were methylmercury and heavy oil. Methylmercury (MeHg) is a natural form of mercury produced by burning fossil fuels and coal that bio-accumulates in food webs and is an endocrine disrupting chemical and neurotoxicant (Alvarez et al., 2006). Routine and burst swimming of pre-flexion croaker (*Micropogonias undulatus*) were significantly reduced upon exposure, and model simulations predicted most exposed larvae would not survive a predator attack (Figure 2.4; Alvarez et al*.,* 2006). Heavy oil causes nervous system damage to early life stages of teleosts, results in chronic behavioural abnormalities, and impacts swimming performance (Irie et al., 2011; Kawaguchi et al., 2011; Johansen et al., 2017) . Pufferfish (*Takifugu rubripes*) larvae displayed abnormal swimming patterns associated with central nervous system defects (Figure 2.4; Kawaguchi et al*.,* 2012). Both of these studies tested pre-flexion stages, which would be most sensitive to these chemicals. Knowledge on how marine fish larvae are affected by toxicants, such as heavy oil, continues to be highly relevant with ongoing use of off-shore oil platforms and underwater drilling activities that have potential spill hazards (e.g., Deepwater Horizon; Johansen & Esbaugh, 2017). Taken together, swimming performance may be a useful metric for ecotoxicological assessment, to measure the direct impact that toxicants have on survivorship of marine fish larvae, regardless of ontogenetic stage.

2.6 Conclusion and Future Work

This chapter provides a comprehensive overview on the biology, physiology, and methodologies characterising swimming performance of marine fish larvae and is framed into an ecological context by assessing the effects of key environmental stressors on larval fish swimming. In summary, I found the following:

I) The larval phase of marine fishes is highly dynamic and influenced by many intrinsic and extrinsic factors that control growth and development. Specific developmental milestones (e.g., notochord flexion) determine when a larva reaches the ability to effectively influence its own dispersal, and I found this competency to be rather temperature- than taxon-dependent. Regardless of taxonomic order, tropical and subtropical fish larvae develop faster and have higher capacity for swimming when compared to temperate fish larvae. Determining the timing of this competency is crucial for improving biophysical dispersal models and assessing connectivity and recruitment scenarios.

II) Several methodologies exist to test the swimming performance of marine fish larvae. They are helpful for investigating different modes of swimming, but their relevance depends on the tested life stage and ecological question. For example, pre-flexion larvae are not competent swimmers, regardless of

taxonomy and climatic region, and are best swum using routine swimming methods. Post-flexion larvae, however, can be tested against continuously increased velocities. For ecological questions related to dispersal distance and connectivity, endurance swimming tests may be the most useful method. Burst swimming tests are valuable for evaluating when and how larvae try to escape predators.

III) I analyzed the effects of key environmental stressors and found that, irrespective of the methodology and climatic region, ocean acidification, ocean warming, and the combination of both have no significant effects on the swimming performance of marine fish larvae. I discuss the potential reasons for the observed trends (e.g., high inter-individual variability, combining data of pre-and post-flexion stages) and provide suggestions for future research. Other stressors, such as reduced prey density or exposure to toxicants, had stronger effects on swimming performance, but depended on the experimental settings (e.g., the duration of prey absence). For example, prey reduction seems to affect prolonged swimming performance more than short-term/acute swimming performance (e.g., critical swimming or burst speeds). In general, I observed a lack of data across all stressors, with respect to developmental stages, methods, climatic regions, and taxonomic groups tested, which hampers better-informed conclusions on the effects of environmental stress on larval fish swimming performance.

Based on my findings, I recommend future studies to address and consider the following points. First, swimming performance should be tested across multiple developmental stages (e.g., both pre-and post-flexion). Currently, swimming performance is often assessed at a single point in time and extrapolated, thus ignoring the rapid changes in performance throughout ontogeny. For example, all recent studies on OA, most OW studies, and OAW have focused on post-flexion larvae. The impacts of these stressors, while not apparent on older larvae, may be more severe earlier in development (e.g., pre-flexion stages). Second, standard protocols for juvenile and adult fishes exist for quantifying the energetic costs of swimming, (e.g., through measurements of oxygen uptake), however, are rare in larval fishes. Future work should aim to add more physiological metrics, which will be invaluable for assessing whether sustaining a certain swimming performance comes with the trade-off of increased energetic costs. While difficult to construct, swim tunnels can be miniaturized enough to assess to the swimming performance of larvae, and fiber optic oxygen probes are available and can detect small changes in water oxygen levels. In more detail, this knowledge could help assess what developmental stages or taxa are most vulnerable to environmental stress, even if no significant differences in swimming performance are observed at first glance. Third, I stress that choosing the right swimming methodology is important: i) the burst swimming method is ideal for evaluating predator escape performance in post-flexion larvae, ii) the routine swimming is a generalist method, and useful for assessing undisturbed swimming in both pre- and post-flexion stages, iii) the U_{crit} method should be used to evaluate short-term, high capacity swimming in

post-flexion fish larvae, and iv) the endurance swimming method is most relevant for providing data that can be used for modelling dispersal and connectivity. All methods are obviously prone to result in different estimates, based on chosen speed increments and time intervals, and I suggest testing these effects to ease comparative approaches. On this note, I caution the use of U_{crit} to model dispersal distances, as larvae likely do not swim at those speeds for a long time; endurance swimming tests are more suited for modelling the dispersal of marine fish larvae in biophysical models. Lastly, I emphasize that testing swimming performance of early life stages of fishes under co-occurring environmental stressors will improve our understanding of how (anthropogenic) environmental stress affects individual fishes and therefore populations. I recommend testing environmental stress on pre-flexion larvae using unforced, routine swimming protocols, as U_{crit} and burst performance methods are more ecologically and physiologically relevant once larvae have further developed. Thus, comparing the swimming performance of larval fishes on an individual level, across developmental stages, taxonomic groups, and climatic regions, provides valuable information as to how population-level dynamics, such as recruitment and connectivity, may be affected by environmental stressors.

This chapter sets the foundation for the remaining chapters of this thesis, by placing the swimming metrics I will use within an environmental context. Additionally, many key knowledge gaps raised in this chapter will be addressed in later chapters, such as measuring oxygen uptake during activity (**chapters 5 and 6**), measuring muscle tissue development (**chapter 4**), and investigating large-scale patterns in larval fish swimming patterns and the factors influencing them (**chapter 3**).

Chapter 3. Habitat association may influence swimming performance in marine teleost larvae

3.1 Summary

Latitude and body size are generally considered key drivers of swimming performance for larval marine fishes, but evidence suggests that evolutionary relationships and habitat may also be important. I used a comparative phylogenetic framework, data-synthesis, and case-study approach to investigate how swimming performance differs among larvae of fish species across latitude. First, I investigated how swimming performance changed with body length, and I found that temperate reef fishes have the greatest increases in swimming performance with length. Secondly, I compared differences in three swimming performance metrics (critical swimming speed, *in situ* swimming, and endurance) among post-flexion larvae, whilst considering phylogenetic relationships, and morphology, and I found that reef fishes have higher swimming capacity than non-reef (pelagic and non-reef demersal) fishes, which is likely due to larger, more robust body sizes. Thirdly, I compared swimming performance of late-stage larvae of tropical fishes with oceanographic data to better understand the ecological relevance of their high-capacity swimming. I found that reef fishes have high swimming performance and grow larger than non-reef fish larvae, which we suggest is due to the pressures to find a specific, patchily-distributed habitat upon which to settle. Given the current bias toward studies on percomorph fishes at low latitudes, I highlight that there is a need for more research on temperate reef fish larvae and other percomorph lineages from high latitudes. Overall, my findings provide valuable context to understand how swimming and morphological traits that are important for dispersal and recruitment processes are selected for among teleost fish larvae.

Associated Publication

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Data availability

Data present in this chapter are available from the Research Data Repository (Tropical Data Hub) at James Cook University. DOI: https://doi.org/10.25903/mqfk-wx96

3.2 Introduction

Chapter 2 introduced the swimming metrics used to swim larvae, and this chapter will place them within a wide ecological context for a wide range of teleost species. This is important because a 2015 study suggested that Marine Protected Areas (MPAs) should encompass approximately 10% of the ocean's surface by the year 2020 (Andrello et al., 2015). Generally, MPAs attempt to preserve marine biodiversity, reduce exploitation of marine resources and assist in the recovery of exploited or disturbed habitats. As such, MPAs represent a conservation and management policy with broad implications for protecting biodiversity, helping coastal human communities, and maintaining healthy stocks that supply young fish to overfished or damaged habitats (Andrello et al., 2015; Harrison et al., 2020). However, to be effective, MPAs must be self-sufficient (e.g., self-recruitment of early life stages) and linked with other marine habitats – protected or not – to promote recovery from disturbances (Planes et al., 2009). Thus, management of the marine environment relies on understanding the spatial scale of the movement of species and their habitat ranges through time (e.g., timing of migrations, movements to nurseries, influx of larvae, etc.; Lowerre-Barbieri et al., 2017; Barbut et al., 2019).

Connectivity among geographically separated marine populations is critical, not only for MPA management, but for general ecosystem function, population structure, genetic diversity, and for improving ecosystem resilience to natural and anthropogenic disturbances (Barbut et al., 2019; Ramos et al., 2017). As highlighted in **chapter 1,** dispersal is a key driver of connectivity and is also a measure of the adaptive capacity of a species to influence habitat range on temporal and spatial scales. For species with bipartite life histories, such as marine teleost fishes (i.e., especially those with site-attached juveniles and adults, such as demersal fishes), dispersal generally occurs during larval stages and is largely influenced by physical processes (e.g., hydrodynamics of the ocean) as well as by individual species traits (Lacroix et al., 2018). Due to the high mortality rates of fish larvae that lead to fluctuations in annual recruitment, dispersal is critical for maintaining population structure/dynamics and resilience to disturbances within fish populations (Lacroix et al., 2018). Several biotic and abiotic factors contribute to differences in dispersal potential among marine fishes, especially at different latitudes. Leis et al., (2013b) describe how several of these factors, such as larval duration, community structure, spawning mode, hydrodynamic conditions, water temperature, and larval behaviour, contribute to latitudinal differences in dispersal potential among coastal fish larvae. The focus of the chapter will test hypothesis 1 of my thesis, by investigating how an influential and universal performance trait among larval fishes – swimming behaviour – differs in relation to the habitat used by the adults (e.g., reef, non-reef demersal, and pelagic) in high and low latitude groups of marine fishes.

As described in **chapter 2**, for most marine teleost fishes, regardless of latitude, locomotor capabilities develop during larval stages (Downie et al., 2020). The focus of many early studies investigating swimming performance of marine teleost larvae was on commercially important pelagic and non-reef demersal species such as herrings (Order Clupeiformes), cods (Order Gadiformes), and flatfishes (Order Pleuronectiformes) that inhabit cold, temperate (high latitude) environments (Miller et al., 1988). These fishes are generally poor swimmers as larvae (<10 cm s^{-1} throughout larval period), and the ability for influential horizontal swimming occurs during late larval or early juvenile life stages (Miller et al., 1988). Early generalisations (e.g., the SA as described in **chapter 1**) suggested that marine fish larvae were planktonic organisms that passively drifted with ocean currents with no influence over where they ended up regardless of latitude, and this had large implications for modelling connectivity and dispersal patterns of larval fishes (e.g., Roberts, 1997). This perspective changed with the discovery that, under laboratory conditions, late-stage tropical coral reef fishes were capable of swimming at high speeds (>40 cm sec^{-1}; Stobutzki & Bellwood, 1994). This was further confirmed with *in situ* observations of actively swimming tropical reef fish larvae (Leis et al., 1996), as well studies that underscored their high endurance capacity (Stobutzki & Bellwood, 1997; Fisher & Bellwood 2001). These findings highlight the potential that tropical reef fishes have to influence their own dispersal. For example, several studies have shown that tropical reef fishes can return to natal reefs (e.g., anemonefishes, Pomacentridae, returning to within a few meters of natal anemones) or disperse using currents and swimming behaviour tens of kilometers to find a new reef home (Jones et al., 1999, 2005; Harrison et al., 2012). Swimming performance among larval fishes in warm tropical waters (i.e., low latitudes) has been considered to be higher compared to fish from cool latitudes because warmer temperatures increase the kinetic reactions within muscle tissues (Fisher et al., 2005; Leis, 2006, 2007). However, recent evidence suggests that larvae of temperate reef fishes are also capable of high swimming speeds (>40 cm s^{-1}; Pattrick & Strydom, 2009; Leis et al., 2012; Faillettaz et al., 2018), despite swimming in 10°C cooler water than larvae of tropical fishes, suggesting that other factors besides temperature may be influencing swimming performance.

Given that evolutionary success is a result of the integration of all life history components across the lifetime of the organism, it is likely that the habitat used by the adults may play a role in larval life history strategies. Both tropical and temperate demersal fishes live in close association with the bottom, but their eggs and larvae are carried by oceanic and reef currents, leaving larvae to develop in the open ocean (Leis, 2006). After a species-specific duration of time, the larvae return to a reef using a combination of current drift and active swimming with and against strong, directional reef currents (Leis, 2006). This contrasts with pelagic fishes that remain in open waters as juveniles and adults after their larval phase, suggesting the environmental pressures associated with swimming may be more consistent across life

history stages. However, larvae of reef fishes have been shown to be better swimmers than larvae of non-reef demersal fishes (e.g., groups like cods and flatfishes that do not settle onto reef habitat). The pressure to find a specific, yet suitable, patchily distributed coral or rocky reef habitat with distinct differences in hydrology (e.g., currents and tidal energy) on which to settle may influence swimming performance in particular groups of reef-associated fishes across latitudes. This hypothesis forms the foundation to the current chapter.

Life history and morphology could play a critical role influencing swimming behaviours among different groups of fishes. For example, oxygen uptake rates among marine fishes scale differently with mass depending on lifestyle (e.g., benthic versus pelagic; Killen et al., 2010). Since larvae of temperate reef fishes swim at impressive swimming speeds (>40 cm s^{-1}; e.g., Leis et al., 2012) when compared to those of pelagic temperate fishes (swimming speed ranged from 10-20 cm s^{-1}; e.g., Miller et al., 1988), interspecific differences in swimming ability may be related to more than previously established factors (e.g., latitude) alone. Body morphology could be one such factor, as body shape has been found to predict swimming performance among tropical reef fish larvae (Fisher & Hogan, 2007). Given the variety of body shapes among different fish groups at the taxonomic level (e.g., circular shapes of Chaetodontidae and ribbon-like forms of Clupeiformes), and how certain families and orders dominate fish communities at specific latitudes (e.g., percomorph fishes are the most abundant group in warm, coastal habitats, and non-percomorphs such as Gadiformes and Clupeiformes are more dominant in cold, pelagic habitats), certain body shapes may be more specialized to high performance swimming. In addition, confirming interspecific differences in performance using phylogenetic comparative methods would show how swimming performance varies among species while accounting for ancestral relationships, and whether the magnitude of swimming ability is correlated with species relatedness. Moreover, such analyses may highlight gaps in knowledge and/or biases from certain taxonomic groups. To date such information is missing from the fish ecology and physiology literature but would provide critical information for managing reserves, as well as understanding the larval dispersal potential for groups of fishes associated with coastal and pelagic habitats.

Here I used comparative phylogenetics, data analysis, and a case-study approach to understand several factors that influence swimming performance in marine teleost fish larvae. Specifically, to test hypothesis 1 of the thesis, which is that the requirement to find a patchily distributed reef habitat shapes high swimming capabilities of marine fish larvae, I take several approaches.

For the first aim (Aim I) I investigated the relationship between swimming ability and growth (using changes in body length as a proxy for growth) across habitats throughout early ontogeny (pre-flexion to post-flexion), using *in situ* and laboratory estimates. I predicted that both tropical and temperate reef-associated fishes would have greater increases in swimming speed with growth when compared to temperate and tropical non-reef fishes in order to achieve faster swimming speeds into later, post-flexion stages. The post-flexion stage represents the point where larvae begin to resemble adult morphologies and swimming ability has the greatest influence on dispersal (Fisher et al., 2000). Therefore, in my second aim (Aim II), I (I.) incorporated phylogenetic comparative analyses to investigate differences in swimming performance among post-flexion larvae from different habitats using three measures of swimming performance (endurance, *in situ*, and critical swimming speed) and (II.) determined the relationship between morphology (body size, body shape, muscle area, and caudal fin area) and swimming performance. I predicted that tropical reef fishes will have the strongest swimming capabilities across all three measures of swimming performance when compared to the other groups, likely due to larger body sizes. Consequently, I predicted that larvae that exhibit more robust body shapes would have higher swimming performance than more slender shaped species. In general, tropical reef fishes have more robust shapes, and therefore I predicted this would support their high swimming performance. Lastly, in my third aim (Aim III), I used a case-study approach to investigate why tropical reef fishes swim to high capacities. I did this by comparing swimming data with oceanographic current data in a representative reef habitat (i.e., Lizard Island, Australia), to place performance within a relevant ecological context. I predicted that tropical reef fishes, which presumably exhibit larger body sizes and robust body shapes, may have high swimming performance in order to surpass fast currents around Lizard Island. Overall, swimming is a critical trait that enables larvae to grow, develop, find a suitable habitat, and ultimately contribute to the supply of new individuals required to support adult populations. My perspective on the role habitat selection may play in influencing swimming performance among fish larvae across latitude may enhance our understanding of dispersal and recruitment strategies of fishes, and our findings will provide critical information for spatial management of fisheries and reserves. As per hypothesis 1 of my thesis, I hypothesized that larvae of reef-associated fishes would have higher swimming abilities compared to their non-reef counterparts and may rely on high growth rates, specialized body plans, and swimming abilities to swim in swiftly-moving waters to find a suitable habitat to settle.

3.3 Methods

3.3.1. Data Collection

3.3.1.1. Literature search

To collect data on marine fish larvae swimming performance, a systematic literature search was performed across several databases (ISI Web of Knowledge, Clarivate Analytics, Core collection) using the following search term: *(swim* OR sust* OR prolong* OR U_{crit} OR in situ) AND (early life stage* OR larv*) AND (marin* OR sea*) AND (fish* OR teleost*)*. Studies were manually checked for appropriateness for my analysis – mainly if the larvae were swum using endurance, *in situ*, or critical swimming speed tests and whether the body length each larva was provided (the exception being endurance swimming, as most studies did not provide body length). Generally, I extracted swimming data directly from the paper or from the supplementary materials on an individual fish level, as most studies expressed swimming as scatter plots (swimming metric vs. size). Swimming data were extracted from the selected studies using WebPlotDigitizer (Version 4.2, Ankit Rohatgi). Other data extracted from the sources included fish length (standard length; mm) and experimental water temperature (°C). We used fish standard length as our proxy for size, as it was the most common metric across all selected studies.

3.3.1.2. Swimming metrics investigated and general data organization

I focused on studies that measured the following swimming metrics: critical swimming speeds (U_{crit}), *in situ* swimming speeds, and endurance capacity. This allowed me to investigate swimming performance of marine teleost larvae over a wide range (e.g., slow to fast performance) of metrics. These are the same swimming metrics described in **chapter 2**, but to refresh, critical swimming speed tests are performed in a laboratory flume using a stepped velocity test, where fish swim against an incrementally faster flow over set time periods until fatigue, and this type of test is an estimate of fast, prolonged swimming speeds that fishes can maintain for short periods of time (Brett, 1964). Of the three investigated swimming metrics, U_{crit} represents the fastest swimming speeds larvae can maintain aerobically (i.e., fatigue endpoint is caused by mainly aerobic and partly anaerobic processes; (Downie & Kieffer, 2017; Illing et al., 2021), and represents the upper limit of their swimming performance that may influence their dispersal capabilities (e.g., overcoming fast currents over a short period of time). *In situ* swimming speeds represent preferred swimming speeds of larvae under natural conditions and are measured by a scuba diving team observing the larvae in the ocean (Leis et al., 1996; and see Leis, 2006 for discussions on potential methodoloigcal biases). Lastly, endurance swimming represents the maximum distance that larvae can swim until fatigue. Generally, fish are forced to swim at a fixed velocity in a laboratory flume until fatigue (e.g., Majoris et al., 2019).

I then grouped each species from our selected studies by 'Environmental Habitat' (Temperate Reef, Temperate Pelagic, Temperate Demersal, Tropical Reef, Tropical Pelagic, and Tropical Demersal), based on each species' description from their respective study. Experimental water temperature was generally confounded with 'Environmental Habitat', with the exception of temperate demersal and temperate pelagic species, which were swum across a wide range of temperatures. Temperate demersal species (e.g., Atlantic cod, *Gadus morhua*, Gadidae; Guan et al., 2008; European plaice, *Pleuronectes platessa*, Pleuronectidae; Silva et al., 2015) from colder habitats were generally swum between 3 and 8.5°C. Some temperate demersal species were from warmer habitats, primarily the East coast of Australia (e.g., Australasian snapper *Pagrus auratus*, Sparidae; Japanese meagre *Argyrosomus japonicus*, Sciaenidae; yellowfin bream *Acanthopagrus australis*, Sparidae; Clark et al., 2005) and were swum between 20 and 23°C and the Gulf of Mexico (e.g., red drum *Sciaenops ocellatus*, Sciaenidae; Faria et al 2009) and were swum at summertime temperatures of 26°C. Temperate pelagic species from colder habitats were swum at 7°C (e.g., Atlantic herring *Clupea harengus*, Clupeidae; Moyano et al 2016) and 15°C (e.g., European pilchard *Sardina pilchurus*, Clupeidae; Silva et al., 2014); whereas, temperate pelagic species from warmer waters were swum at 17.5°C (e.g., sand smelt *Atherina presbyter*, Atherinidae; Faria et al., 2014) and 20°C (e.g., alewife *Alosa pseudoharengus*; Clupeidae; Klumb et al., 2003). Temperate reef fishes were swum within a narrow range of temperatures, spanning 18.5 to 20°C. Tropical fishes (pelagic, demersal, and reef) were swum between 26 and 30°C.

The criteria used to classify a species as 'reef-associated' was whether they settled to adopt a close association with a benthic coral (tropical) or rocky coastal (temperate) habitat after a pelagic phase as a larva. Demersal fishes are fishes that settle in association with benthic habitats that are not reefs (e.g., gravel, muddy, sandy bottoms) after a pelagic larval phase. Pelagic fishes remain in the water column across ontogenetic stages (i.e., larvae to adult). I classified each individual larva in the entire analysis based on ontogenetic stage, either pre-notochord flexion or post-notochord flexion (which also contained flexion-stage larvae), as described in **chapter 2**. Pre-notochord flexion larvae generally have underdeveloped swimming fins and muscles and have limited ability to control horizontal movement (Downie et al., 2020). Over a narrow species-specific length range, the notochord flexes, and the caudal fin forms, marking an increase in horizontal swimming ability (post-notochord flexion; Downie et al., 2020). These classifications were based on when this transition between pre- and post-notochord flexion stages occurs, as per each species' body length, which was indicated by their respective studies.

3.3.2 AIM I: Development of swimming performance over growth

3.3.2.1 Data organisation

Swimming speed across length (standard length; mm) was collected from marine teleost larvae, focussing on U_{crit} and *in situ* metrics, as not enough studies measured endurance swimming over development. I only included studies that measured swimming over the entire larval duration (pre- and post-flexion) of the study species, versus focusing on one stage (e.g., studies that exclusively swam post-flexion fish). All six 'Environmental Habitats' were analysed for U_{crit}; however, 'Temperate Reef' and 'Temperate Pelagic' species were not present in the literature for *in situ* analyses.

3.3.2.2. Statistical analyses

All statistical analyses for Aim I were performed using R (ver 3.6.1. http://www.R-project.org/). Prior to statistical analyses, data were checked for normality using visual diagnostic tests (Q-Q plots) and the Shapiro-Wilkes test. The swimming data (U_{crit} and *in situ* swimming speeds) and body length were log-transformed prior to analyses for normality. Linear models, and 'emtrends' function from the 'emmeans' package (Lenth, 2019), were used to compare slopes between 'Environmental Habitats' as swimming speed changed with body length. 'Environmental Habitat' was considered a categorical variable in the model, swimming speed (U_{crit} or *in situ*) was a response variable, and body length was a continuous variable. The most appropriate model tested swimming speed (U_{crit} or *in situ*) as a function of the interaction of 'Environmental Habitat' (categorical) and body length (continuous). This model was selected based on the lowest Akaike Information Criterion (AIC) using the 'MuMIn' package (Barton, 2009). Upper and lower confidence intervals were determined using the 'emmeans' package using the best fitting model. We set an α=0.05 to compare slopes between 'Environmental Habitats'.

3.3.3. AIM II. Comparing swimming performance of post-flexion larvae

3.3.3.1. data organisation

I used post-flexion stage larvae from all six 'Environmental Habitats' and three swimming metrics – U_{crit}, *in situ*, and endurance – for analyses. I expressed U_{crit} and *in situ* swimming in absolute (cm sec^{-1}) terms. For endurance data, I only included studies that had swum fish at similar flow speeds (10-13 cm s^{-1}) and did not feed their larvae during the experiment, so that the studies would be comparable.

At the post-flexion stage, fish larvae exhibit a wide range of body sizes, which is confounded with 'Environmental Habitat', as tropical species are generally larger than temperate species. I investigated the influence of body length on swimming performance among my 'Environmental Habitats'

in two different ways. The first model investigated differences in swimming performance over all sizes, with the exception being endurance swimming tests because body length data were not readily available. Generally, covariates should be within a similar range, and data outside that range should be omitted from the analysis (Quinn & Keogh, 2002). Thus I created a second model, where I sub-set the body length data to compare U_{crit} among my 'Environmental Habitats' within a range of sizes common among all groups (16-22mm SL) because there are large size differences between groups (e.g., tropical reef, versus temperate pelagic and temperate demersal groups). I could not perform similar analyses on in situ swimming, as there was not enough overlap in body sizes (Figure 3.7).

3.3.3.2. body shape analyses

To perform the analyses on body morphology and swimming performance, I extracted images from the literature (e.g., the studies that swum the fish), larval fish guides, and online sources. For any particular post-flexion stage individual or representative species, I found images that were of similar size (<5mm difference between the size of the image and size of the fishes swum in their respective study). I was able to find images for 80% of the species of post-flexion stage larvae within an appropriate size range, and on average, the difference between the size of the individual swum and the images used for body shape analyses was 1.5mm ± 1.5mm (mean ± standard deviation). Following the procedures of Fisher & Hogan, (2007), I measured total length, body depth, body area, propulsive area, muscle area, caudal fin height, and caudal fin area in ImageJ (Figure 3.1). I was then able to calculate body shape as the ratio of body length to body depth, following Fisher & Hogan, (2007). While I measured propulsive area and body area, these metrics did not add to the model and were omitted from the analyses, but I kept the data in the raw data files for body shape.

3.3.3.3. statistical analyses

Model diagnostics were the same as in Aim I. I then created size distributions for each 'Environmental Habitat' to highlight the differences in sizes (length, mm) at settlement between the groups. Bin size (4mm) was uniform across 'Environmental Habitats'. First, linear models were used to compare swimming performance (U_{crit}, *in situ*, or endurance) between 'Environmental Habitats' as a function of body length. 'Environmental Habitats' were considered fixed, categorical factors in the model, and values for swimming performance (U_{crit}, *in situ*, and endurance) and body length were continuous variables. For U_{crit}, two models were tested: U_{crit} in response to the interaction of 'Environmental Habitat' across the complete body length range of all species and U_{crit} in response to the interaction of 'Environmental Habitat' and at a common body length range of 16-22 mm SL across all 'Environmental

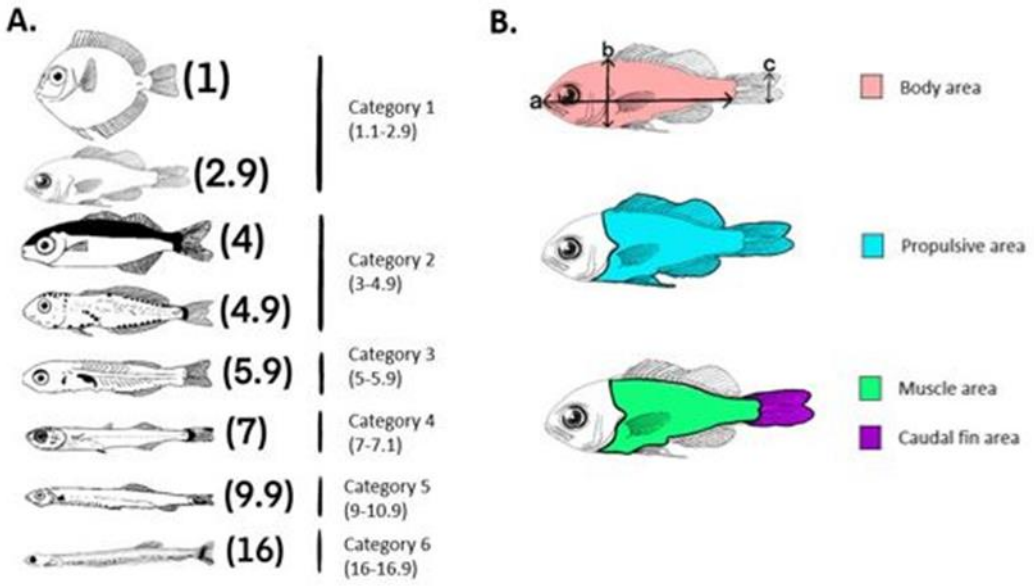

Figure 3.1 Images showing body shapes and measurements of morphology to predict swimming capacity among fishes in our analyses. **A.** visual representation of an individual within each body shape category. Body shape was calculated as body length/body depth from reference photos for each species within a narrow size range (see Methods 3.3.3.2 for details). The large number in brackets is the body shape for that particular photo, and each category indicates the numerical body shape range within it. **B.** A visual representation of the morphological features measured in each photo: *a* standard length, *b* body depth, and *c* caudal fin depth. From each photo, I also measured body area, propulsive area, muscle area, and caudal fin area in order to calculate the ratio of propulsive area and muscle area to body area, and ration of caudal depth to caudal area (see Methods 3.3.3.2 for details).

Habitats'. For *in situ* swimming speed, only one model could be tested: *in situ* swimming speed in response to the interaction of 'Environmental Habitat' and body length (i.e., all body lengths). For endurance swimming, only one model could be tested: endurance distance in response to 'Environmental Habitat', as body length data were not widely available. Second, a linear model was used to test U_{crit} in response to the interaction between 'Environmental Habitat' and body shape. Third, linear models were used to test for differences in morphology (i.e., muscle area and caudal fin area) in response to 'Environmental Habitat' as a function of body length. For both muscle area and caudal fin area, two models were tested: muscle or caudal fin area in response to 'Environmental Habitat' (all body lengths) and muscle or caudal fin area in response to the interaction of 'Environmental Habitat' and a common body length range from 16-22 mm SL. Morphology metrics (body shape, muscle area, caudal fin area) were all continuous variables. For body shape, swimming performance, morphology, and body length were log-transformed for statistical analyses (as data were not normally distributed) but then back-transformed to the response scale for plotting figures. The most appropriate model, including an interaction with body length, was selected based on the lowest Akaike Information Criterion (AIC) using the 'MuMIn' package (Barton, 2009). For all swimming tests (U_{crit}, *in situ*, and endurance) and body shape analyses versus 'Environmental Habitat' analyses, estimated marginal means were calculated using the 'emmeans' package (Lenth, 2019) based on the best fit model and provided p-values from all our models. Significant differences were determined using a Tukey's post hoc test (α=0.05).

3.3.3.4. comparative phylogenetic analyses

Comparative phylogenetic methods were performed in R (R Development Core Team, 2013) using the 'ape' (Paradis & Schliep, 2019) and 'phytools' (Revell, 2012) packages. I used a pre-existing phylogenetic tree representing all extant fishes for the analyses accessed using the 'fishtree' package (Chang et al., 2019; Rabosky et al., 2018). The scientific names of the species selected were validated using the rfishbase package (Boettiger et al., 2012), and I pruned the entire tree based on these species. I pruned the phylogenetic tree down to tips that represented species sampled for each swimming metric (U_{crit}, *in situ*, or endurance swimming) using the function 'drop.tip' from the ape package. 'Environmental Habitat' was considered a fixed factor, and swimming speed (U_{crit}, *in situ*) or swimming distance (endurance) and body length were continuous factors. The best model for the phylogenetic comparative analyses, including an interaction with body length, was selected based on the lowest AIC rankings using the 'MUMIn' package. A Phylogenetic General Least Squares (PGLS; α=0.05) model ('nlme' package; Pinheiro *et al.,* 2019) was used to investigate patterns of variation in swimming performances between species from different 'Environmental Habitats' while accounting for phylogeny. Trees and their

associated physiological traits (U_{crit}, *in situ*, and endurance swimming) were plotted using the 'ggtree' package (Yu et al., 2017, 2018).

3.3.4. AIM III: Case study: swimming performance over ontogeny versus Lizard Island current velocities

3.3.4.1. Swimming performance over ontogeny of three demersal spawning reef fishes versus Lizard Island current velocities

I used swimming data from (Fisher et al., 2000) for this first objective. In their study, they measured U_{crit} daily over the entire larval duration from hatch until the point of settlement of three tropical reef fishes, ambon damsefish (*Pomacentrus amboinensis,* Pomacentridae), cinnamon anemonefish (*Amphiprion melanopus*, Pomacentridae*)*, and pajama cardinal fish (*Sphaeramia nematoptera,* Apogonidae*)*. The two pomacentrid species (*P. amboinensis* and *A. melanopus*) have demersal eggs; whereas, the apogonid (*S. nematoptera*) has orally brooded eggs. For the analysis, I first calculated Reynold's Number (*Re*) for each U_{crit} measurement per species (see **chapter 2** for description of *Re* and the formula). This allowed me to estimate when each species was capable of swimming under an inertial swimming regime ($Re > 1000$) and therefore when swimming would be expected to have some capacity to influence dispersal potential. Johansen, (2014) measured currents at three distinct reef habitats (i.e., exposed, oblique, and sheltered reefs at three replicate sites per habitat) around Lizard Island on the northern Great Barrier Reef, Australia, using a custom-built flow meter at depths of 3 and 9 m below the surface. The three reef habitats vary with respect to physical cover from the south-easterly trade winds, with 'exposed reefs' having little cover, 'oblique reefs' having some cover, and 'sheltered reefs' having ample cover from the winds (see Johansen, (2014) for a schematic of the reefs). The depth range measured by Johansen, (2014) represents a range of depths at which reef fishes swim when they approach the reef (see review by Leis, 2006). Current speeds were measured every 10 seconds, and on average, the current meter at each replicate site (3) per habitat per depth recorded 251±47 h (mean ± standard error; s.e.) of current data (Johansen, 2014). I used the averaged current speed for each habitat (averaged across the three replicate sites) for the depths of 3 and 9 m (i.e., as per Figure 4 in Johansen, 2014) for my case study. I superimposed the averaged annual current speed from each habitat and depth onto the swimming speed data (see Figures 3.12 and 3.13 in Results). I could then compare the ages of larvae swimming under inertial regimes, factoring the average flow rates around the island at different depths, to infer whether remaining near the reef during the larval stage would be feasible.

3.3.4.2. Swimming performance of settlement-stage coral reef fishes versus Lizard Island current speeds

Following a similar approach to (Fisher, 2005), I used the same previously described oceanographic data from 3.3.4.1, and superimposed it onto the speeds of different swimming metrics (i.e., estimated minimum swimming speed, average *in situ* speed, and average U_{crit}) of settlement stage tropical coral reef fishes. Swimming data were adapted from (Leis & Fisher, 2006) in which U_{crit} and *in situ* speeds were measured in 30 species of settlement-stage reef fish larvae. To understand whether reef fishes swim at or beyond the minimum requirements of their habitats prior to settlement, I first had to define 'minimum' for each species. To do this, I calculated, for each species, the swimming speed at which *Re* would be 1000 (i.e., the lowest *Re* at which an animal is predicted to overcome the viscosity of water). I then combined these data with average *in situ* speed (cm s^{-1}) and average U_{crit} for species within each family. Superimposing the swimming data on to the oceanographic data (Johansen, 2014) would then provide an estimate as to whether *in situ* or maximum swimming is sufficient to swim against reef currents at specific depths for each habitat and at which speeds, relative to minimum performance, fishes operate. I included swimming speeds for a tropical pelagic fish species and a tropical demersal fish species, where *in situ* and U_{crit} were available for the same species, as a form of comparison with the reef fishes.

3.4 Results

3.4.1. Search results

The literature search resulted in 37 relevant studies for our analyses. My analyses, across all three aims, comprised 209 species (including 19 species only to genus level, and 26 species only to Family level) from 44 families in 25 orders of teleost fishes (Table 3.1).

3.4.2. AIM I: Development of swimming performance over growth

Correlation between body size and critical swimming speed was highest among temperate (r^2=0.80) and tropical (r^2=0.64) reef fishes. (Figure 3.2). In contrast, temperate pelagic fishes had the narrowest range in U_{crit} relative to length, and swimming speed was poorly correlated with body size (r^2=0.0056; Figure 3.2). Temperate reef fishes had the highest increase in U_{crit} with increasing body size ($\text{Log}(U_{crit})$=2.7Log(Length)-4.25; Figure 3.2), and temperate pelagic fishes were found to have the lowest increase in U_{crit} with increasing body size ($\text{Log}(U_{crit})$=0.29Log(Length)+0.6; Figure 3.2). The relationship between increasing body length and U_{crit} was generally different between all groups, except for temperate demersal and tropical demersal (p=0.99), temperate demersal and tropical pelagic (p=0.78), tropical demersal and tropical pelagic (p=0.73), and tropical demersal and tropical reef (p=0.77) (Figure 3.2; also,

Table 3.1 Summary of taxonomic groups (Order and Family level) used for the analyses in this study (AIMS I, II and III), organized into 'Environmental Habitat' (Tropical Reef, Tropical Pelagic, Tropical Demersal, Temperate Reef, Temperate Pelagic, Temperate Demersal), swimming metrics (critical swimming speed U_{crit}, *in situ*, and endurance), and the number of species swum for each metric.

Order	Family	Environmental Habitat	U_{crit} (G)	*In situ* (G)	U_{crit} (PF)	*In situ* (PF)	End. (PF)
Acanthuriformes	Acanthuridae	Tropical Reef	0	0	5	0	0
Atheriniformes	Atherinidae	Temperate Pelagic	1	0	1	0	0
Blenniiformes	Blenniidae	Tropical Reef	0	0	4	0	0
	Grammatidae	Tropical Reef	1	0	1	0	0
	Pomacentridae	Tropical Reef	3	0	40	0	30
		Temperate Reef	1	0	1	0	0
Carangiformes	Carangidae	Tropical Reef	0	0	2	0	1
		Tropical Pelagic	2	1	2	1	1
	Rachycentridae	Tropical Reef	0	0	1	0	0
Centrarchiformes	Perichthyidae	Temperate Demersal	0	0	0	0	1
	Terapontidae	Tropical Reef	0	0	1	0	0
Chaetodontiformes	Chaetodontidae	Tropical Reef	0	0	8	0	10
	Leiognathidae	Tropical Demersal	1	1	1	1	1
Clupeiformes	Clupeidae	Temperate Pelagic	1	0	3	0	0
		Tropical Pelagic	0	0	2	0	0
Ephippiformes	Ephippidae	Tropical Reef	1	1	1	1	0
Gadiformes	Gadidae	Temperate Demersal	1	0	1	0	0
Gobiesociformes	Gobiesocidae	Temperate Reef	2	0	0	0	0
Gobiiformes	Gobiidae	Tropical Reef	2	0	2	0	0
Gonorynchiformes	Chanidae	Tropical Pelagic	1	0	1	0	0
Holocentriformes	Holocentridae	Tropical Reef	0	0	2	0	0
Istophoriformes	Polynemidae	Tropical Demersal	1	1	1	1	1
Kurtiformes	Apogonidae	Tropical Reef	1	0	22	0	1
Labriformes	Labridae	Tropical Reef	0	0	7	0	0
Lobotiformes	Moronidae	Temperate Reef	1	0	1	0	0
	Sciaenidae	Temperate Demersal	3	2	3	2	1
Lophiiformes	Ogcocephalidae	Tropical Reef	0	0	1	0	0
Lutjaniformes	Haemulidae	Tropical Reef	0	0	1	0	0

	Lutjanidae	Tropical Reef	1	1	10	1	0
Perciformes	Psychrolutidae	Temperate Demersal	1	0	1	0	0
	Sebastidae	Temperate Reef	5	0	0	0	0
	Serranidae	Tropical Reef	4	2	5	2	0
Pleuronectiformes	Pleuronectidae	Temperate Demersal	1	0	1	0	0
Scombriformes	Nomeidae	Tropical Pelagic	0	0	1	0	0
Spariformes	Lethrinidae	Tropical Reef	0	0	1	0	0
	Nemipteridae	Tropical Reef	0	0	2	0	0
	Pomacanthidae	Tropical Reef	0	0	2	0	0
	Siganidae	Tropical Reef	0	0	1	0	0
	Sparidae	Temperate Reef	5	0	10	0	1
		Temperate Demersal	2	1	2	1	2
		Tropical Reef	1	0	0	0	0
Syngnathiformes	Mullidae	Tropical Reef	0	0	1	0	1
	Sphyraenidae	Tropical Reef	0	0	1	0	0
	Syngnathidae	Tropical Reef	0	0	1	0	0
Tetraodontiformes	Balistidae	Tropical Reef	0	0	2	0	0
	Diodontidae	Tropical Reef	0	0	1	0	0
	Monacanthidae	Temperate Reef	0	0	0	0	1
		Tropical Reef	0	0	6	0	0
	Ostraciidae	Tropical Reef	0	0	2	0	0
	Tetraodontidae	Tropical Reef	0	0	3	0	0

G= U_{crit} and *In situ* over growth studies (AIM I), PF= Post-Flexion larvae used for U_{crit}, *in situ,* and endurance studies (AIM II and III).

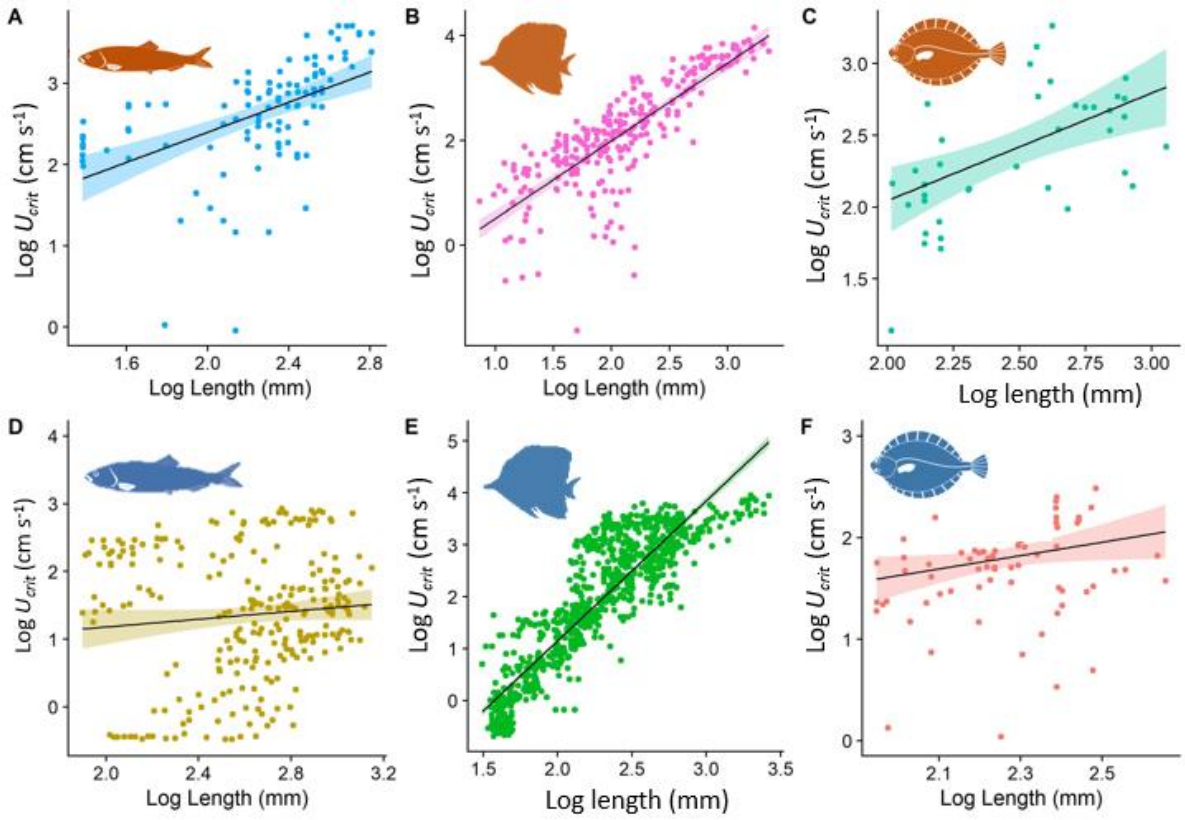

Figure 3.2 Changes in critical swimming speed (U_{crit}; cm s^{-1}) with length (standard length; mm) for marine teleost fishes from six 'Environmental Habitats': **A.** tropical pelagic, **B.** tropical reef, **C.** tropical demersal, **D.** temperate pelagic, **E.** temperate reef, **F.** temperate demersal. A butterflyfish symbol represents reef fish species, the herring symbol represents pelagic fish species, and the flatfish symbol represents demersal fish species. Blue colored symbols represent temperate species, and orange-coloured symbols represent tropical species. Raw data are included onto each panel respective to 'Environmental Habitat' and represent an individual larva. The regression equations for each 'Environmental Habitat' are based on best fitting model (refer to supplementary materials), and the equations are as follows: tropical pelagic: U_{crit} = 0.93Length-0.97 r^2=0.26; tropical reef: U_{crit} = 1.48Length-0.97 r^2=0.64; tropical demersal: U_{crit} = 1.25Length-0.38 r^2=0.51; temperate pelagic: U_{crit} = 0.29Length+0.6 r^2=0.0056; temperate reef: U_{crit} = 2.7Length-4.25 r^2=0.8; temperate demersal: U_{crit} = 1.16Length-0.6 r^2 = 0.35.

Table 3.2 Summary statistics for the linear model describing the relationship between U_{crit}, 'Environmental Habitat' (temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef) and body length (mm)

Model: lm(log(Ucm)~Habitat*log(length), data)

	Est.	**Std. error**	**T**	**p**
(Intercept)	-0.6	0.12	-5.1	4.51e-07
HabitatTempPel	1.2	0.36	3.3	0.00089
HabitatTempReef	-3.7	0.16	-22.2	<2e-16
HabitatTropDem	0.21	0.4	0.55	0.58
HabitatTropPel	1.1	0.4	2.8	0.0047
HabitatTropReef	-0.38	0.19	-1.9	0.054
Log(length)	1.2	0.056	20.8	<2e-16
HabitatTempPel: log(length)	-0.87	0.14	-6.2	7.12e-10
HabitatTempReef:log(length)	1.5	0.076	20.4	<2e-16
HabitatTempDem:log(length)	0.09	0.17	0.54	0.59
HabitatTropPel:log(length)	-0.23	0.18	-1.3	0.19
HabitatTropReef:log(length)	0.32	0.092	3.5	0.00042

$F_{11, 2417}$=391

Table 3.3 Pairwise comparisons, using a Tukey's post-hoc test, of the changes in U_{crit} with body length for six 'Environmental Habitats' (temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef)

	Est.	**Std.error**	**Df**	**t**	**p**
Temp Dem X Temp Pel	0.086	0.014	2417	6.2	<0.0001
Temp Dem X Temp Reef	-0.15	0.0075	2417	-20.4	<0.0001
Temp Dem X Trop Dem	-0.0089	0.017	2417	-0.54	0.99
Temp Dem X Trop Pel	0.023	0.018	2417	1.3	0.78
Temp Dem X Trop Reef	-0.032	0.0091	2417	-3.5	0.0056
Temp Pel X Temp Reef	-0.24	0.014	2417	-17.4	<0.0001
Temp Pel X Trop Dem	-0.095	0.02	2417	-4.7	<0.0001
Temp Pel X Trop Pel	-0.063	0.021	2417	-3	0.034
Temp Pel X Trop Reef	-0.12	0.014	2417	-8.1	<0.0001
Temp Reef X Trop Dem	0.14	0.016	2417	8.7	<0.0001
Temp Reef X Trop Pel	0.18	0.018	2417	10	<0.0001
Temp Reef X Trop Reef	0.12	0.0088	2417	13.7	<0.0001
Trop Dem X Trop Pel	0.032	0.023	2417	1.4	0.73
Trop Dem X Trop Reef	-0.023	0.017	2417	-1.4	0.77
Trop Pel X Trop Reef	-0.055	0.018	2417	-3	0.03

see Table 3.2 and Table 3.3 for details on statistical output). However, the way that *in situ* swimming changes with increasing size was similar between groups (i.e., tropical reef, tropical pelagic, tropical demersal, and temperate demersal), as slopes were not significantly different (Figure 3.3; $p>0.3$ for all combinations of groups; refer to Table 3.4 and Table 3.5 for details on statistical output).

3.4.3. AIM II. Comparing swimming performance of post-flexion larvae

Upon reaching post-flexion, tropical reef fishes are generally larger than larvae from other 'Environmental Habitats' (Figure 3.4). Among the tropical reef fishes from the available studies, are the largest individuals and, overall, larger size classes with the highest frequency of individuals. Among the temperate reef fishes at or beyond flexion stages there are higher frequency of larger size classes than in temperate pelagic and demersal fishes (Figure 3.4).

When considering all sizes of post-flexion larvae, tropical reef fishes have the highest critical swimming speed (speeds (U_{crit}; Figure 3.5a). Temperate pelagic fishes had the lowest U_{crit} values of all 'Environmental Habitats' (Figure 3.5a). Temperate reef fishes swim faster than tropical pelagic fishes ($p=0.036$) but not significantly different than tropical demersal fishes ($p=0.79$; refer to Table 3.6 and Table 3.7 for details statistical output; Figure 3.5a). Comparing U_{crit} within a similar size range (16-22mm SL) revealed that tropical reef, tropical demersal, and temperate reef fishes swim at similar speeds ($p>0.1$ for all combinations; refer to Table 3.8 and Table 3.9 for details on statistical output; Figure 3.5b).

Upon accounting for phylogenetic relationships among species (PGLS), I found significant differences in U_{crit} between settlement stage larvae with respect to environmental habitat and body length ($p<0.0001$ and $p<0.0001$, respectively; Figure 3.6, Table 3.10). The majority of reef-associated fishes for which data exist on swimming larvae, especially in the tropical latitudes, are from Percomorpha; whereas, larvae from temperate regions include species from the non-percomorph orders (e.g., Clupeiformes and Gadiformes Figure 3.6).

Similar to larvae used in U_{crit} experiments, larvae swum for *in situ* experiments from tropical reef habitats were larger at post-flexion stages, and a higher frequency of larger size classes were represented when compared to larvae from temperate non-reef and tropical non-reef habitats (Figure 3.7). Temperate larvae were generally smaller and showed a higher frequency of smaller size classes than tropical fishes (Figure 3.7). Among post-flexion larvae swimming *in situ*, we found that tropical reef fish larvae were swimming significantly faster than the other groups of fishes ($p<0.03$ for all combinations; for details on statistical output refer to Figure 3.8). I did not find a significant effect of length ($p=0.92$) or 'Environmental Habitat' ($p=0.51$) on *in situ* swimming performance when accounting

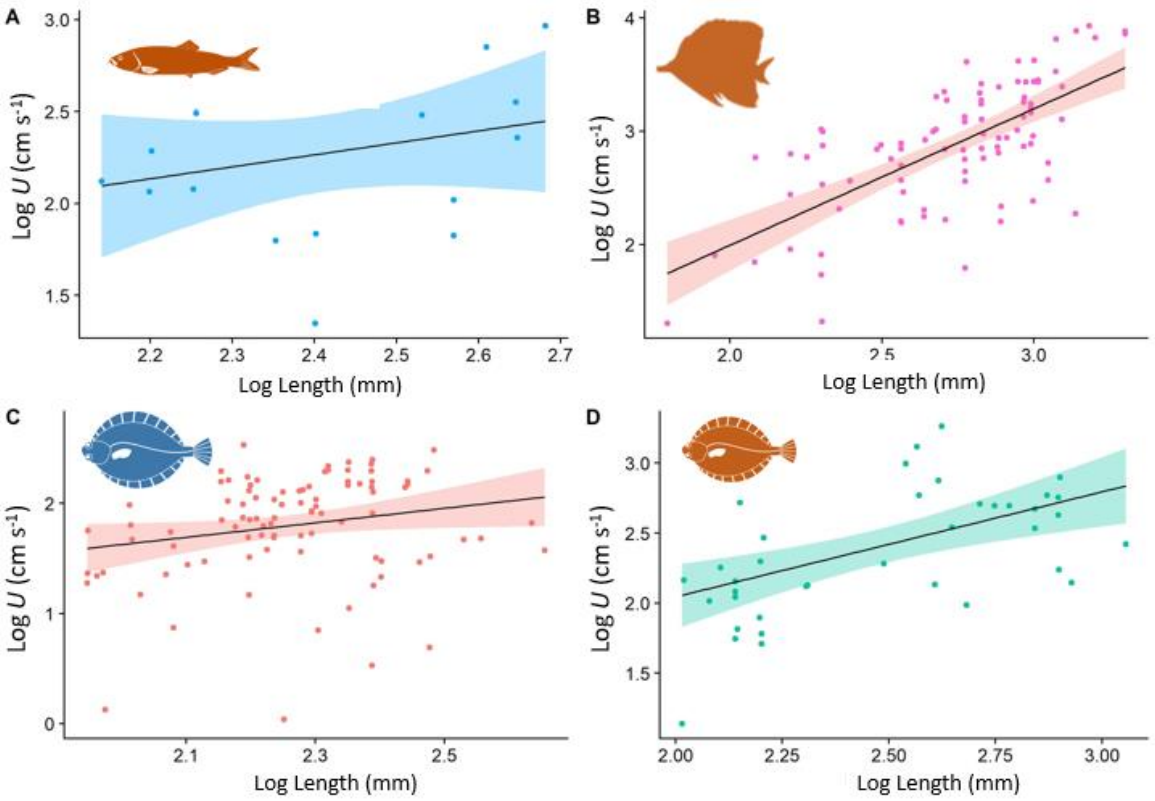

Figure 3.3 Changes in *in situ* swimming (*U*; cm s^{-1}) with length (standard length; mm) for marine teleost fishes from four Environmental Habitats': **A.** tropical pelagic, **B.** tropical reef, **C.** temperate demersal, **D.** tropical demersal). A butterflyfish symbol represents reef fish species, a herring symbol represents pelagic fish species, and a flatfish symbol represents demersal fish species. A blue colored symbol represents temperate species, and an orange coloured symbol represents tropical species. Raw data are included onto each panel respective to 'Environmental Habitat' and represent an individual larva. The regression equations for each 'Environmental Habitat' are based on best fitting model (refer to supplementary materials), and the equations are as follows: tropical pelagic: $U = 0.65\text{Length}+0.7$ $r^2=0.02$; tropical reef: $U = 1.2\text{Length}-0.42$ $r^2=0.44$; temperate demersal: $U = 0.66\text{Length}+0.31$ $r^2=0.034$; tropical demersal: $U = 0.75\text{Length}+0.55$ $r^2=0.25$.

Table 3.4 Summary statistics for the linear model describing the relationship between *in situ* swimming and body length between fish larvae from four 'Environmental Habitats' (temperate demersal, tropical pelagic, tropical demersal, tropical reef)

Model: lm(log(Ucm)~Habitat*log(length), data)

	Est.	**Std. error**	**t**	**p**
(Intercept)	0.31	0.67	0.46	0.64
HabitatTropDem	0.24	0.87	0.27	0.79
HabitatTropPel	0.39	1.6	0.25	0.81
HabitatTropReef	-0.73	0.78	-0.93	0.35
Log(length)	0.65	0.3	2.2	0.027
HabitatTropDem:log(length)	0.092	0.37	0.25	0.8
HabitatTropPel:log(length)	-0.0068	0.666	-0.01	0.99
HabitatTropReef:log(length)	0.55	0.33	1.7	0.099

Table 3.5 Pairwise comparisons, using a Tukey's post-hoc test, of the changes in *in situ* swimming speed with body length for four 'Environmental Habitats' (temperate demersal, tropical pelagic, tropical demersal, tropical reef)

	Est.	**Std.error**	**df**	**t**	**p**
Temp Dem X Trop Dem	-0.0073	0.029	236	-0.25	0.99
Temp Dem X Trop Pel	0.00054	0.053	236	0.01	1
Temp Dem X Trop Reef	-0.043	0.026	236	-1.7	0.35
Trop Dem X Trop Pel	0.0078	0.05	236	0.16	0.99
Trop Dem X Trop Reef	-0.036	0.021	236	-1.7	0.32
Trop Pel X Trop Reef	-0.044	0.049	236	-0.9	0.8

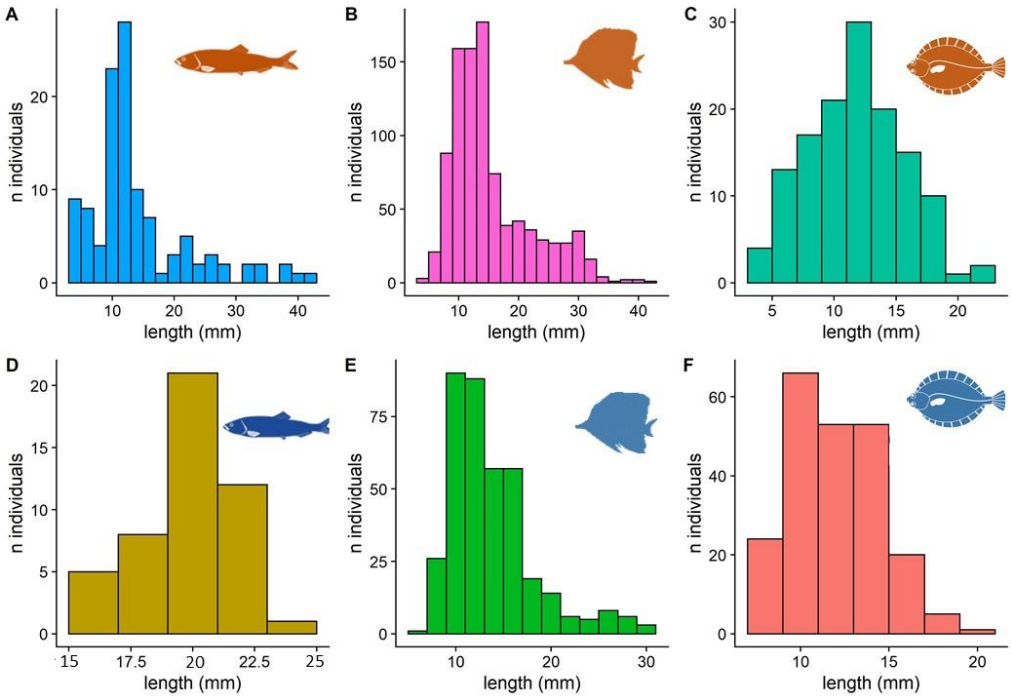

Figure 3.4 Size distributions (n individuals) for settlement stage larvae used in U_{crit} experiments from **A.** tropical pelagic, **B.** tropical reef, **C.** tropical demersal, **D.** temperate pelagic, **E.** temperate reef, and **F.** temperate demersal 'Environmental Habitats'. A butterflyfish symbol represents reef fish species, the herring symbol represents pelagic fish species, and a flatfish symbol represents demersal fish species. A blue coloured symbol represents temperate species, and an orange-coloured symbol represents tropical species.

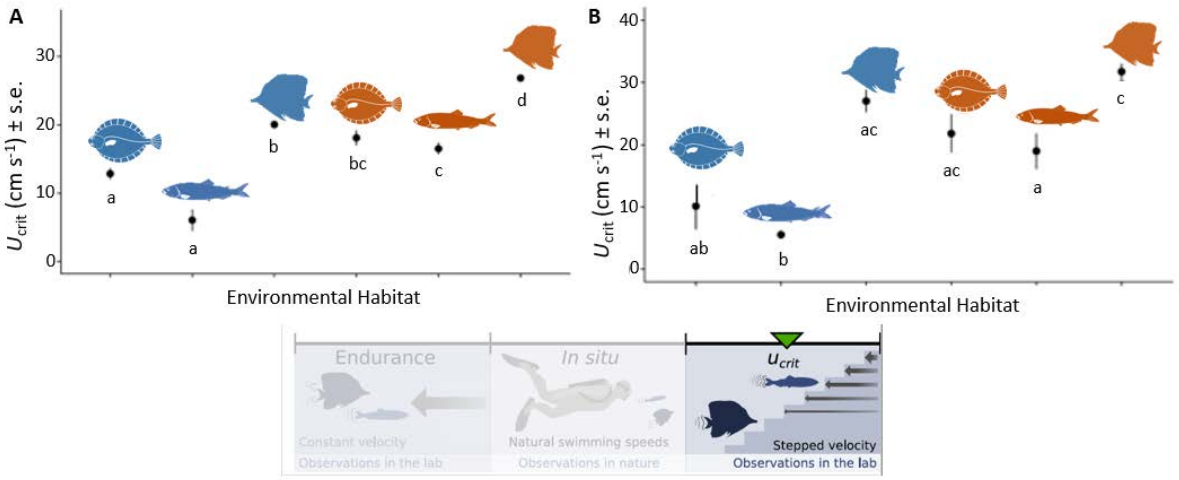

Figure 3.5 Average critical swimming speed (mean U_{crit}; cm s^{-1}; ± standard error, s.e.) of post-flexion stage marine teleost larvae from six 'Environmental Habitats', based on latitude (orange = tropical, blue = temperate) and habitat (herring symbol = pelagic, butterflyfish symbol = reef, flatfish symbol = demersal) **A.** comparing swimming speeds of post-flexion larvae across all sizes and **B.** comparing swimming speeds at a common size range (16-22mm SL). Linear models included body length (standard length; mm) as a covariate, and different lower-case letters represent statistical differences between groups (α=0.05).

Table 3.6 Summary statistics for the linear model between U_{crit} and habitat association of post-flexion larvae from the six 'Environmental Habitats'(temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), taking body length (mm) into account

Model=lm(log(Ucm)~Habitat * log(length), data)

	Est.	**Std.error**	**t**	**p**
(Intercept)	2.7	0.19	14.3	2e-16
HabitatTempPel	-0.68	0.96	-0.7	0.48
HabitatTempReef	-0.74	0.21	-3.44	0.0006
HabitatTropDem	-1.4	0.25	-5.7	1.52e-08
HabitatTropPel	-0.19	0.22	-0.87	0.38
HabitatTropReef	-0.085	0.2	-0.43	0.67
Log(length)	-0.015	0.016	0.94	0.35
HabitatTempPel:log(length)	-0.0051	0.05	-0.1	0.92
HabitatTempReef:log(length)	0.082	0.017	4.7	2.33e-06
HabitatTropDem:log(length)	0.12	0.02	6	2.36e-09
HabitatTropPel:log(length)	0.03	0.017	1.8	0.077
HabitatTropReef:log(length)	0.056	0.016	3.5	0.00049

$F_{11,1826}$=101.8

Table 3.7 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in U_{crit} between post-flexion larvae from six 'Environmental Habitats'(temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), taking body length (mm) into account

	Est.	**Std.error**	**df**	**t**	**p**
Temp Dem X Temp Pel	2.1	0.58	1826	2.7	0.069
Temp Dem X Temp Reef	0.65	0.04	1826	-6.9	<0.0001
Temp Dem X Trop Dem	0.71	0.058	1826	-4.2	0.0005
Temp Dem X Trop Pel	0.78	0.06	1826	-3.2	0.019
Temp Dem X Trop Reef	0.49	0.028	1826	-12.6	0.9111
Temp Pel X Temp Reef	0.31	0.083	1826	-4.3	0.0002
Temp Pel X Trop Dem	0.34	0.093	1826	-3.9	0.0011
Temp Pel X Trop Pel	0.37	0.1	1826	-3.6	0.004
Temp Pel X Trop Reef	0.23	0.062	1826	-5.5	<0.0001
Temp Reef X Trop Dem	1.1	0.074	1826	1.3	0.79
Temp Reef X Trop Pel	1.2	0.074	1826	2.9	0.036
Temp Reef X Trop Reef	0.74	0.026	1826	-8.4	<0.0001
Trop Dem X Trop Pel	1.1	0.089	1826	1.2	0.84
Trop Dem X Trop Reef	0.68	0.044	1826	-6	<0.0001
Trop Pel X Trop Reef	0.62	0.035	1826	-8.4	<0.0001

Table 3.8 Summary statistics for the linear model between U_{crit} and habitat association of post-flexion larvae from the six 'Environmental Habitats'(temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), at a common body length (16-22mm)

Model: lm(log(Ucm)~Habitat * log(length), data)

	Est.	**Std.error**	**t**	**p**
(Intercept)	1.9	8.9	0.21	0.83
HabitatTempPel	0.89	9.3	0.096	0.92
HabitatTempReef	-0.93	9.2	-0.1	0.92
HabitatTropDem	-0.3	9.6	-0.003	0.99
HabitatTropPel	15.7	9.6	1.6	0.1
HabitatTropReef	0.18	9.04	0.02	0.98
Log(length)	0.15	3.2	0.048	0.96
HabitatTempPel:log(length)	-0.51	3.3	-0.16	0.88
HabitatTempReef:log(length)	0.65	3.2	0.202	0.84
HabitatTropDem:log(length)	0.27	3.4	0.08	0.93
HabitatTropPel:log(length)	-5.1	3.4	-1.5	0.13
HabitatTropReef:log(length)	0.33	3.2	0.102	0.92

$F_{11,299}$=47.3

Table 3.9 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in U_{crit} between post-flexion larvae from six 'Environmental Habitats' (temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), at a common body length (16-22mm)

	Est.	**Std.error**	**df**	**t**	**p**
Temp Dem X Temp Pel	1.8	0.68	299	1.6	0.59
Temp Dem X Temp Reef	0.38	0.14	299	-2.7	0.089
Temp Dem X Trop Dem	0.46	0.18	299	-1.9	0.36
Temp Dem X Trop Pel	0.53	0.21	299	-1.6	0.6
Temp Dem X Trop Reef	0.32	0.12	299	-3.1	0.024
Temp Pel X Temp Reef	0.21	0.022	299	-15.1	<0.0001
Temp Pel X Trop Dem	0.25	0.041	299	-8.5	<0.0001
Temp Pel X Trop Pel	0.29	0.05	299	-7.1	<0.0001
Temp Pel X Trop Reef	0.18	0.016	299	-19.4	<0.0001
Temp Reef X Trop Dem	1.2	0.19	299	1.4	0.74
Temp Reef X Trop Pel	1.4	0.24	299	2.1	0.28
Temp Reef X Trop Reef	0.85	0.067	299	-2.03	0.33
Trop Dem X Trop Pel	1.2	0.24	299	0.67	0.98
Trop Dem X Trop Reef	0.69	0.1	299	-2.6	0.11
Trop Pel X Trop Reef	0.6	0.094	299	-3.3	0.016

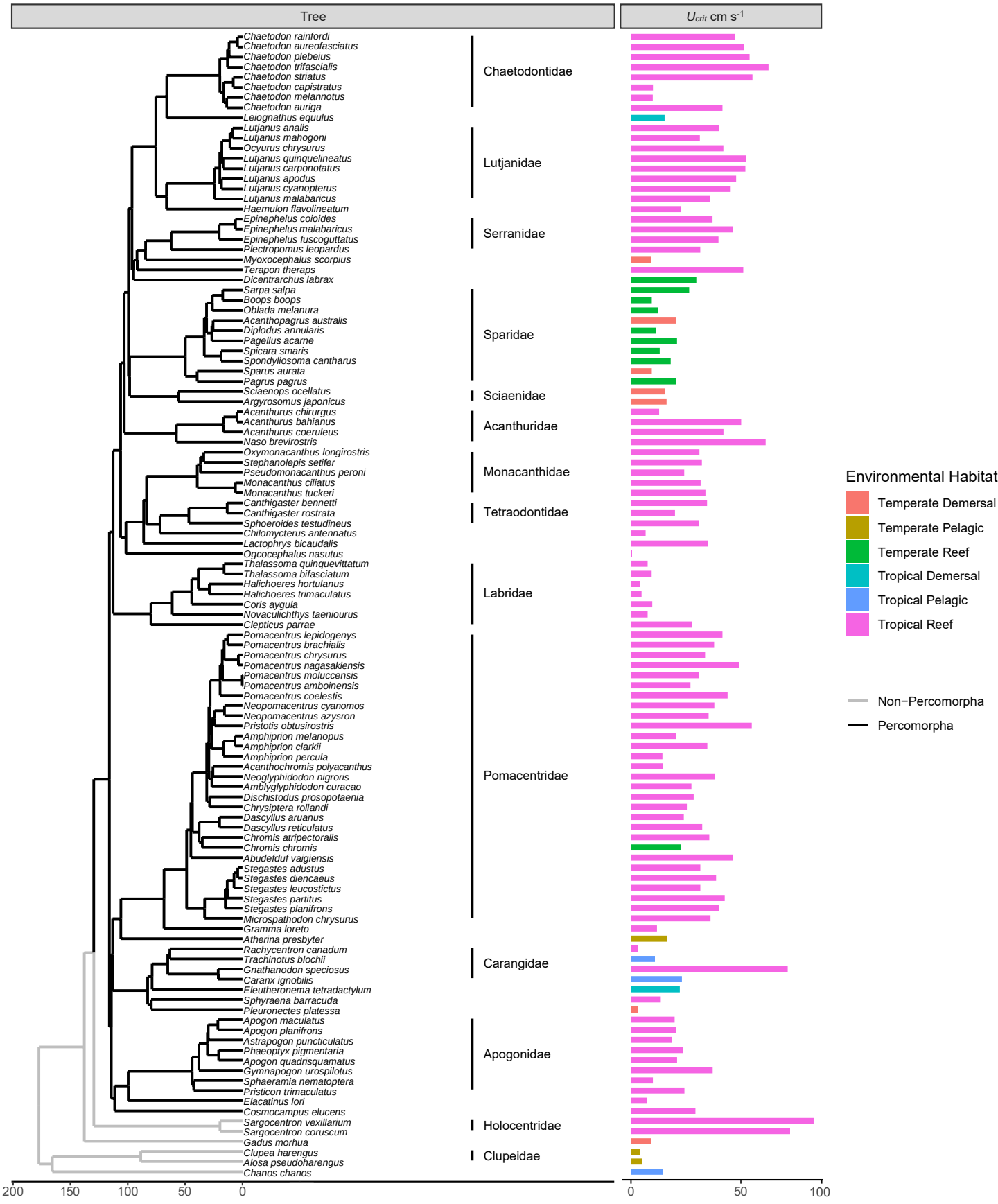
Tree
U_{crit} cm s^{-1}
Chaetodon rainfordi
Chaetodon aureofasciatus
Chaetodon plebeius
Chaetodon trifascialis
Chaetodon striatus
Chaetodon capistratus
Chaetodon melannotus
Chaetodon auriga
Leiognathus equulus
Lutjanus analis
Lutjanus mahogoni
Ocyurus chrysurus
Lutjanus quinquelineatus
Lutjanus carponotatus
Lutjanus apodus
Lutjanus cyanopterus
Lutjanus malabaricus
Haemulon flavolineatum
Epinephelus coioides
Epinephelus malabaricus
Epinephelus fuscoguttatus
Plectropomus leopardus
Myoxocephalus scorpius
Terapon theraps
Dicentrarchus labrax
Sarpa salpa
Boops boops
Oblada melanura
Acanthopagrus australis
Diplodus annularis
Pagellus acarne
Spicara smaris
Spondyliosoma cantharus
Sparus aurata
Pagrus pagrus
Sciaenops ocellatus
Argyrosomus japonicus
Acanthurus chirurgus
Acanthurus bahianus
Acanthurus coeruleus
Naso brevirostris
Oxymonacanthus longirostris
Stephanolepis setifer
Pseudomonacanthus peroni
Monacanthus ciliatus
Monacanthus tuckeri
Canthigaster bennetti
Canthigaster rostrata
Sphoeroides testudineus
Chilomycterus antennatus
Lactophrys bicaudalis
Ogcocephalus nasutus
Thalassoma quinquevittatum
Thalassoma bifasciatum
Halichoeres hortulanus
Halichoeres trimaculatus
Coris aygula
Novaculichthys taeniourus
Clepticus parrae
Pomacentrus lepidogenys
Pomacentrus brachialis
Pomacentrus chrysurus
Pomacentrus nagasakiensis
Pomacentrus moluccensis
Pomacentrus amboinensis
Pomacentrus coelestis
Neopomacentrus cyanomos
Neopomacentrus azysron
Pristotis obtusirostris
Amphiprion melanopus
Amphiprion clarkii
Amphiprion percula
Acanthochromis polyacanthus
Neoglyphidodon nigroris
Amblyglyphidodon curacao
Dischistodus prosopotaenia
Chrysiptera rollandi
Dascyllus aruanus
Dascyllus reticulatus
Chromis atripectoralis
Chromis chromis
Abudefduf vaigiensis
Stegastes adustus
Stegastes diencaeus
Stegastes leucostictus
Stegastes partitus
Stegastes planifrons
Microspathodon chrysurus
Gramma loreto
Atherina presbyter
Rachycentron canadum
Trachinotus blochii
Gnathanodon speciosus
Caranx ignobilis
Eleutheronema tetradactylum
Sphyraena barracuda
Pleuronectes platessa
Apogon maculatus
Apogon planifrons
Astrapogon puncticulatus
Phaeoptyx pigmentaria
Apogon quadrisquamatus
Gymnapogon urospilotus
Sphaeramia nematoptera
Pristicon trimaculatus
Elacatinus lori
Cosmocampus elucens
Sargocentron vexillarium
Sargocentron coruscum
Gadus morhua
Clupea harengus
Alosa pseudoharengus
Chanos chanos
Chaetodontidae
Lutjanidae
Serranidae
Sparidae
Sciaenidae
Acanthuridae
Monacanthidae
Tetraodontidae
Labridae
Pomacentridae
Carangidae
Apogonidae
Holocentridae
Clupeidae
200
150
100
50
0
0
50
100
Environmental Habitat
Temperate Demersal
Temperate Pelagic
Temperate Reef
Tropical Demersal
Tropical Pelagic
Tropical Reef
Non−Percomorpha
Percomorpha

Figure 3.6 Time calibrated phylogeny of fishes (modified from Rabosky et al 2018) and the distribution of average critical swimming speed (U_{crit}) of post-caudal fin flexion stage teleost larvae from six 'Environmental Habitats' (Tropical Reef, Tropical Demersal, Tropical Pelagic, Temperate Reef, Temperate Demersal, Temperate Pelagic). Percomorph lineages are indicated by black lines and non-percomorph lineages are indicated by gray lines.

Table 3.12 Phylogenetic general least square analysis (PGLS) of the U_{crit} of post-flexion larvae in relation to 'Environmental Habitat' (temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef) and body length (mm)

Model=gls(Ucm~Habitat+length, correlation=corBrownian(phy=tree),data, method="ML")

	numDF	**F-value**	**P**
(Intercept)	1	3.13	0.079
Habitat	5	53.21	<0.0001
Length	1	72.3	<0.0001

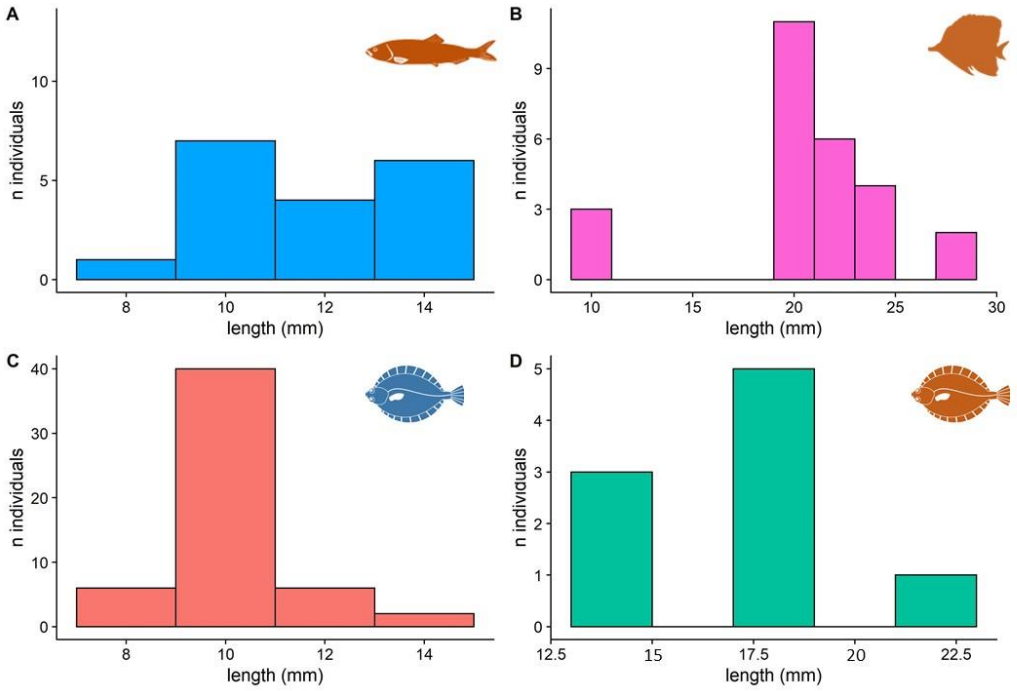

Figure 3.7 Size distributions for settlement stage larvae used in *in situ* swimming observations from A) tropical pelagic, **B.** tropical reef, **C.** temperate demersal, and **D.** tropical demersal 'Environmental Habitats'. A butterflyfish symbol represents reef fish species, the herring symbol represents pelagic fish species, and a flatfish symbol represents demersal fish species. A blue coloured symbol represents temperate species, and an orange coloured symbol represents tropical species.

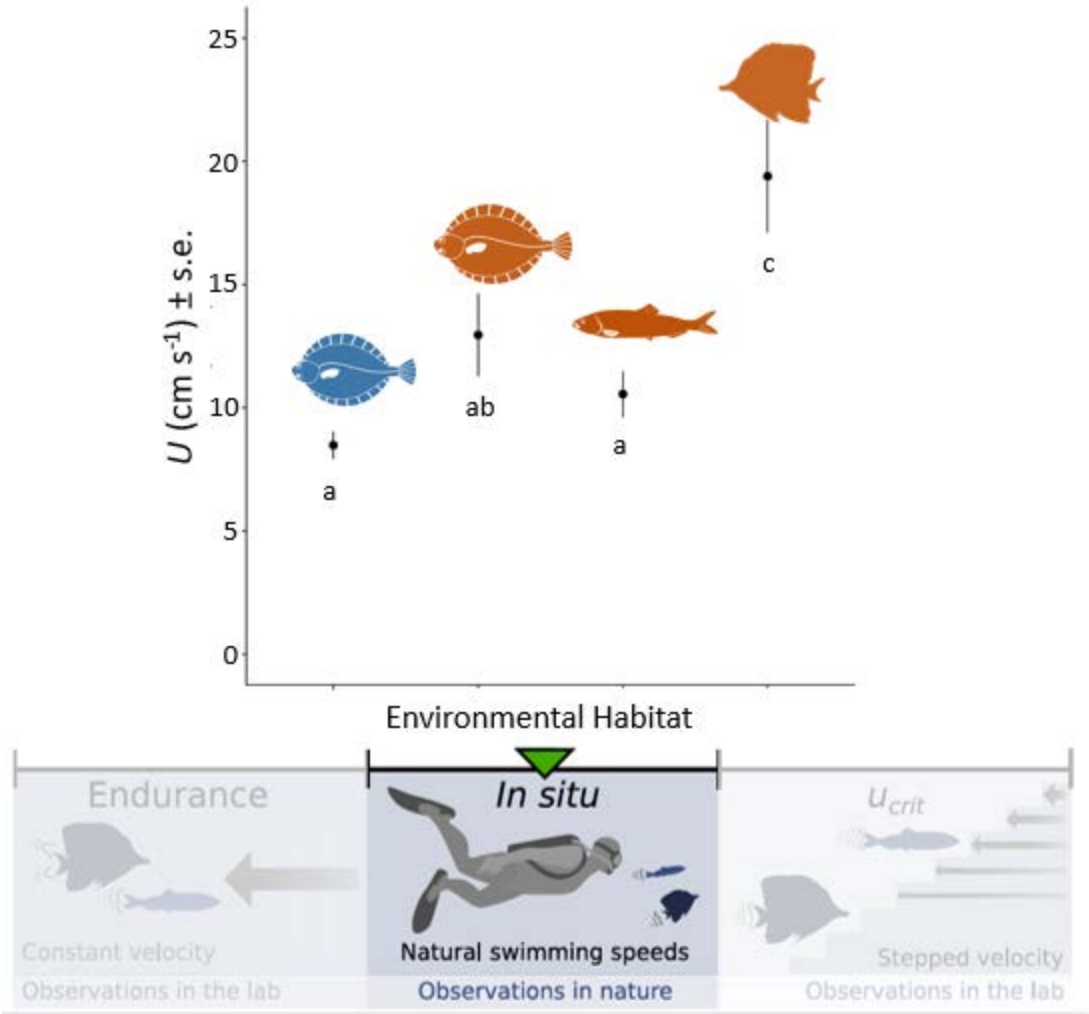

Figure 3.8 Average *in situ* swimming speed (mean *U*; cm s^{-1} ± standard error, s.e.) of post-flexion stage marine teleost larvae from four 'Environmental Habitats', based on latitude (orange = tropical, blue = temperate) and habitat (herring symbol = pelagic, butterflyfish symbol = reef, flatfish symbol = demersal). Linear models included body length (standard length; mm) as a covariate, and different lower-case letters represent statistical differences between groups (α=0.05).

Table 3.11 Summary statistics for the linear model between *in situ* swimming and habitat association of post-flexion larvae from the six 'Environmental Habitats' (temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), taking body length (mm) into account

lm(log(Ucm)~Habitat*log(length), data)

	Est.	**Std.error**	**t**	**p**
(Intercept)	1.7	0.38	4.5	1.58e-05
HabitatTropDem	2.7	0.84	3.3	0.0014
HabitatTropPel	-0.14	0.61	-0.23	0.82
HabitatTropReef	0.36	0.5	0.71	0.48
Log(Length)	0.029	0.037	0.79	0.43
HabitatTropDem:log(length)	-0.13	0.057	-2.3	0.022
HabitatTropPel:log(length)	0.033	0.055	0.6	0.55
HabitatTropReef:log(length)	0.029	0.04	0.71	0.48

$F_{7,99}$= 36.51

Table 3.12 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in *in situ* swimming between post-flexion larvae from six 'Environmental Habitats', taking body length into account

	Est.	**Std.error**	**df**	**t**	**p**
TempDem X TropDem	0.65	0.1	102	-2.6	0.044
TempDem X TropPel	0.8	0.08	102	-2.2	0.13
TempDem X TropReef	0.44	0.071	102	-5.1	<0.0001
TropDem X TropPel	1.2	0.2	102	1.2	0.6
TropDem X TropReef	0.67	0.098	102	-2.8	0.035
TropPel X TropReef	0.54	0.09	102	-3.7	0.0021

for phylogeny (refer to Table 3.13 for details on statistical output). Across all three 'Environmental Habitats' from this subset of analyses, the fishes were exclusively percomorphs.

While temperate reef fish larvae at flexion stages were on average capable of swimming for longer distances (average=30.8±11.9 km) at constant speeds (10-13 cm s^{-1}) than fish from other groups, there were no statistical differences in endurance swimming between temperate reef fishes, tropical reef fishes (24.98±3.1km), tropical demersal fishes (14.32±2.4km), and tropical pelagic fishes (16.13±4.3km) ($p>0.05$; Figure 3.9, Table 3.14, 3.15). Temperate demersal fishes had less endurance capacity than fishes from all of the other 'Environmental Habitats' ($p<0.0001$; Figure 3.9, Table 3.14, 3.15). Similar to my findings with *in situ* swimming performance, I found no significant effect of 'Environmental Habitat' ($p=0.47$) when I accounted for phylogenetic relatedness (Table 3.16).

Fishes with more robust body morphologies typically had higher swimming performance than thinner, more ribbon-like species (Figure 3.10). Tropical reef fishes largely exhibited more robust body shapes than other groups, and as consequence, had higher swimming performance than the species from the other habitats (Figure 3.10). Temperate demersal fishes and tropical pelagic species ($p=0.13$) as well as temperate reef fishes and tropical demersal fishes ($p=0.42$) had similar swimming capabilities and similar body shapes (Figure 3.10), which were less robust than tropical reef species (for details on statistical output, refer to Table 3.17 and Table 3.18; Figure 3.10). Temperate pelagic species had the most elongated body shapes, and as consequence, had the poorest swimming capabilities (Figure 3.10). Post-flexion larvae of tropical reef fishes have high muscle and caudal fin area across all body sizes when compared to fishes from the other 'Environmental Habitats' ($p<0.0001$ for all combinations; refer to Table 3.19, Table 3.20, Table 3.23, Table 3.24 for details on statistical output; Figure 3.11). However, when comparing at a common body size (16-22mm SL), there were no difference in these metrics between tropical reef and tropical pelagic fishes (refer to Table 3.21, Table 3.22, Table 3.25, Table 3.26 for details on statistical analyses; Figure 3.11).

3.4.4. AIM III: Case study: swimming performance over ontogeny and at settlement versus Lizard Island current velocities

3.4.4.1. Swimming performance over ontogeny versus Lizard Island current velocities

The three reef fish species (i.e., *Pomacentrus amboinensis, Amphiprion melanopus*, and *Sphaeramia nematoptera*) where U_{crit} was measured in laboratory-reared larvae across days post hatch (dph) attained influential swimming abilities (*Re* >1000) at very different ages. *P. amboinensis* became capable of influential swimming at 12dph (~11 cm s^{-1}; ~7.5mm TL; settlement at 20 dph; Figure 3.12). *A. melanopus* became capable of influential swimming at 6dph (~12 cm s^{-1}; 8.05mm TL; settlement at 9dph;

Table 3.13 Phylogenetic general least square analysis (PGLS) of the *in situ* swimming of post-flexion larvae in relation to 'Environmental Habitat' (temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef) and body length (mm)

Model= gls(Ucm~Habitat+length, correlation=corBrownian(phy=tree),data, method="ML")

	numDF	F-value	P
(Intercept)	1	9.76	0.035
Environment Habitat	2	0.79	0.51
Length	1	0.011	0.92

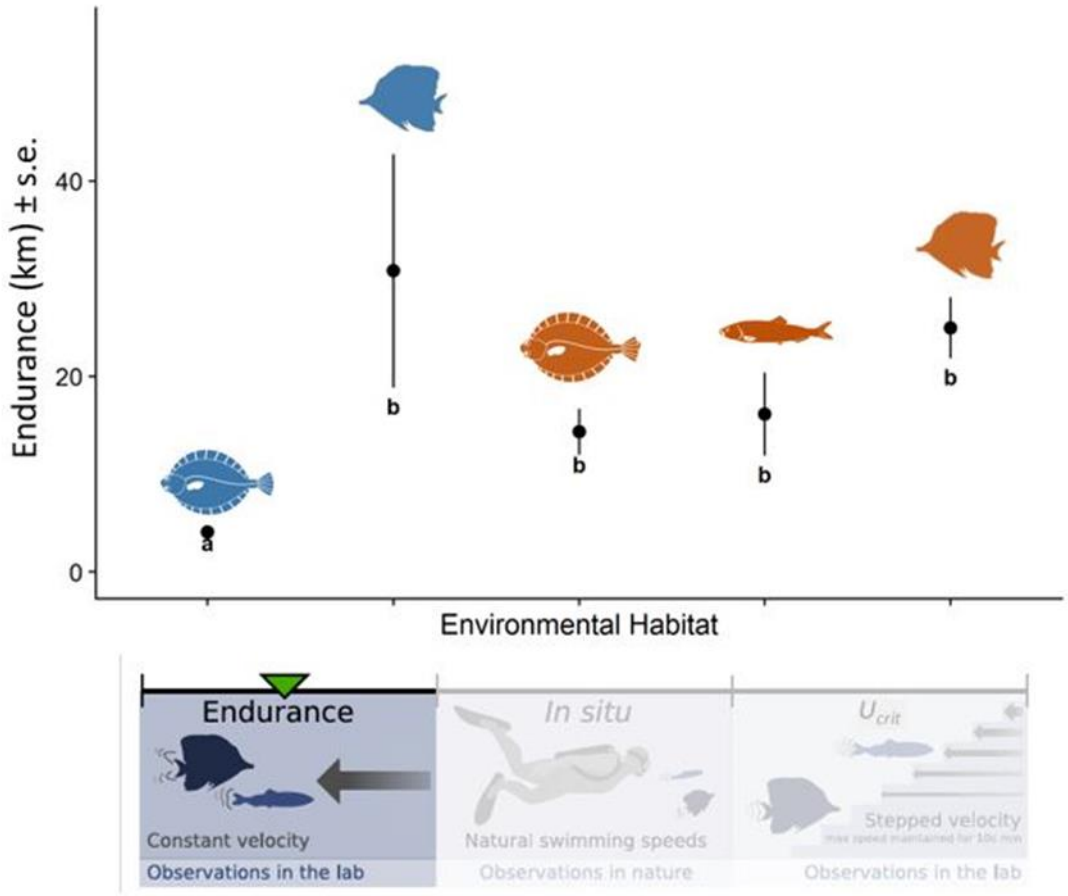

Figure 3.9 Endurance distance (km ± standard error, s.e.) of settlement-stage marine teleost larvae from five 'Environmental Habitats', based on latitude (orange = tropical, blue = temperate) and habitat (herring symbol = non-reef, butterflyfish symbol = reef, flatfish symbol = demersal). All larvae were swum at fixed speeds of 10-13 cm s^{-1}. Different lower-case letters represent statistical differences between groups (α=0.05).

Table 3.14 Summary statistics for the linear model between endurance swimming and habitat association of post-flexion larvae from the five 'Environmental Habitats' (temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef)

Model=lm(endurance~Habitat, family=gamma(link='log'), data)

	Est.	**Std.error**	**T**	**p**
(Intercept)	1.4	0.12	11.5	<2e-16
HabitatTempReef	2.03	0.41	5	1.25e-06
HabitatTropDem	1.3	0.2	6.2	3.19e-09
HabitatTropPel	1.4	0.29	4.8	3.82e-06
HabitatTropReef	1.8	0.17	10.4	<2e-16

Table 3.15 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in endurance swimming between post-flexion larvae from five 'Environmental Habitats'(temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef)

	Est.	**Std.error**	**df**	**t**	**p**
TempDem X TempReef	0.13	0.053	Inf	-5	<0.0001
TempDem X TropDem	0.28	0.058	Inf	-6.2	<0.0001
TempDem X TropPel	0.25	0.073	Inf	-4.8	<0.0001
TempDem X TropReef	0.16	0.028	Inf	-10.4	<0.0001
TempReef X TropDem	2.2	0.91	Inf	1.8	0.36
TempReef X TropPel	1.9	0.9	Inf	1.4	0.64
TempReef X TropReef	1.2	0.5	Inf	0.51	0.99
TropDem X TropPel	0.89	0.28	Inf	-0.38	0.99
TropDem X TropReef	0.57	0.12	Inf	-2.7	0.056
TropPel X TropReef	0.65	0.19	Inf	-1.5	0.57

Table 3.16 Phylogenetic general least square analysis (PGLS) of the endurance swimming of post-flexion larvae in relation to 'Environmental Habitat' (temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef) and body length

Model=gls(Endurance~Habitat, correlation=corBrownian(phy=tree),data, method="ML")

	numDF	**F-value**	**P**
(Intercept)	1	9.76	0.014
Environment Habitat	2	0.794	0.47

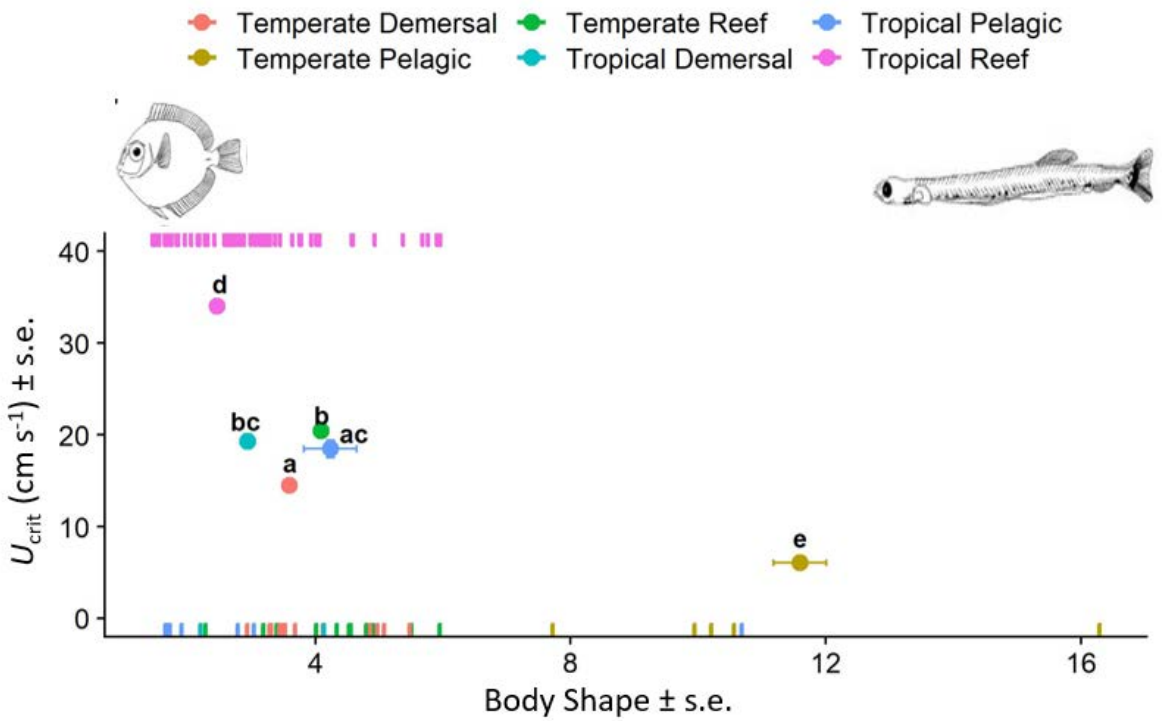

Figure 3.10 Average critical swimming speed (mean U_{crit}; cm s^{-1} ± standard error; s.e.) of post-flexion stage marine teleost fishes from six 'Environmental Habitats' (temperate demersal, temperate reef, temperate pelagic, tropical demersal, tropical reef, tropical pelagic) versus body shape (ratio of body length to body width). Raw data (x-axis for tropical pelagic, tropical demersal, temperate reef, temperate demersal, and temperate pelagic habitats; tropical reef data placed above figure to mitigate cluttering of data points) show the spread of the body shapes per 'Environmental Habitat'. A representative example of most robust body shape (surgeonfish Acanthuridae, left side of figure; body shape ratio [body length:body depth] =1) and most stream-lined body shape (herring Clupeidae, right side of figure; body shape ratio [body length:body depth] =16) are present on the figure to help visualise the shapes (refer to Figure 3.1 for full range of body shapes). Linear models were used to analyse data, and different lower-case letters represent statistical differences between groups (α=0.05).

Table 3.17 Summary statistics for the linear model between U*crit*, body shape, and habitat association of post-flexion larvae from six 'Environmental Habitats' (temperate demersal, temperate reef, temperate pelagic, tropical pelagic, tropical demersal, tropical reef)

lm(log(Ucm)~log(elongation)+Habitat, data)

Note: Elongation: Short for body shape, calculated as the ratio of body height to body length

	Est.	**Std. error**	**T**	**P**
Intercept	1.8	0.13	13.3	<2e-16
Log(elongation)	-0.38	0.043	-8.8	<2e-16
NicheTemperatePelagic	-0.45	0.094	-4.8	2.05e-6
NicheTemperateReef	0.34	0.043	7.9	4.3e-15
NicheTropicalDemersal	0.21	0.069	3.08	0.0021
NicheTropicalPelagic	0.16	0.064	2.5	0.014
NicheTropicalReef	0.67	0.045	14.9	<2e-16

$F_{6,1185}$=144.9

Table 3.18 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in U_{crit}, and body shape between post-flexion larvae from six 'Environmental Habitats' (temperate pelagic, temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef)

	ratio	**SE**	**df**	**t.ratio**	**P**
Temperate Demersal X Temperate Pelagic	1.6	0.15	1185	4.8	<0.0001
Temperate Demersal X Temperate Reef	0.71	0.031	1185	-7.9	<0.0001
Temperate Demersal X Tropical Demersal	0.81	0.056	1185	-3.1	0.026
Temperate Demersal X Tropical Pelagic	0.86	0.054	1185	-2.5	0.1324
Temperate Demersal X Tropical Reef	0.51	0.023	1185	-14.9	<0.0001
Temperate Pelagic X Temperate Reef	0.45	0.041	1185	-8.8	<0.0001
Temperate Pelagic X Tropical Demersal	0.52	0.058	1185	-5.9	<0.0001
Temperate Pelagic X Tropical Pelagic	0.55	0.058	1185	-5.7	<0.0001
Temperate Pelagic X Tropical Reef	0.33	0.033	1185	-10.9	<0.0001
Temperate Reef X Tropical Demersal	1.1	0.077	1185	1.9	0.4214
Temperate Reef X Tropical Pelagic	1.2	0.073	1185	3.01	0.0313
Temperate Reef X Tropical Reef	0.72	0.031	1185	-7.8	<0.0001
Tropical Demersal X Tropical Pelagic	1.1	0.085	1185	-7.8	0.9807
Tropical Demersal X Tropical Reef	0.63	0.041	1185	-6.9	<0.0001
Tropical Pelagic X Tropical Reef	0.59	0.036	1185	-8.6	<0.0001

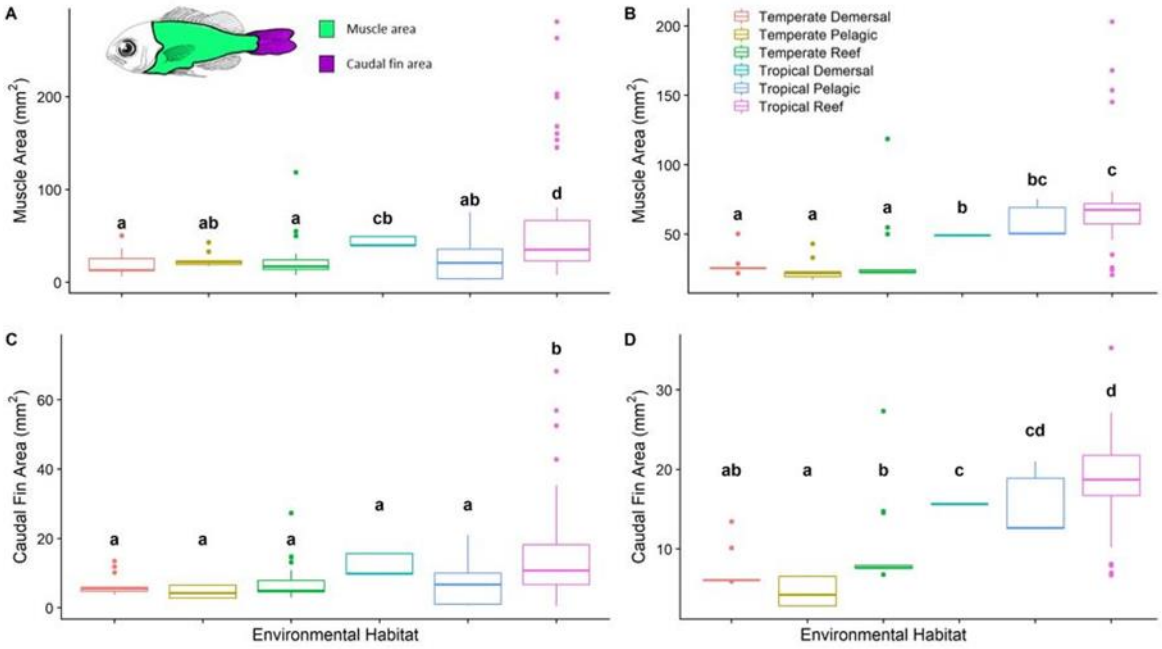

Figure 3.11 Comparison of morphological predictors of swimming performance among six 'Environmental Habitats' (tropical reef, tropical pelagic, tropical demersal, temperate reef fish, temperate pelagic, temperate demersal). Muscle area (mm^2) and caudal fin area for each group across all size ranges (A. for muscle and C. for caudal fin) and among a common size range (16-22mm) (B. for muscle area and D. for caudal fin area). Different lower-case letters represent statistical differences between groups (α=0.05).

Table 3.19 Summary statistics for the relationship between U_{crit} and muscle area (mm^2) for post-flexion larvae from six 'Environmental Habitats'(temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef)

Model=lm(Muscle.area~Habitat, data)

	Est.	**Std. error**	**T**	**p**
Intercept	16.9	2.6	6.6	7.05e-11
HabitatTempPel	6.2	6.2	1.01	0.31
HabitatTempReef	3.2	3.3	0.99	0.32
HabitatTropDem	26.6	5.3	5	6.35e-07
HabitatTropPel	5.7	4.9	1.2	0.24
HabitatTropReef	44.5	3.2	13.9	<2e-16

$F_{5,1186}$=63.23

Table 3.20 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in U_{crit} between post-flexion larvae from six 'Environmental Habitats'(temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), and muscle area (mm^2).

	Ratio	**SE**	**Df**	**t**	**p**
TempDem X TempPel	-6.2	6.2	1186	-1.01	0.9136
TempDem X TempReef	-3.3	3.3	1186	-0.99	0.9211
TempDem X TropDem	-26.6	5.3	1186	-5	<0.0001
TempDem X TropPel	-5.7	4.9	1186	-1.2	0.85
TempDem X TropReef	-44.5	3.2	1186	-13.9	<0.0001
TempPel X TempReef	2.9	5.9	1186	0.5	0.9961
TempPel X TropDem	-20.4	7.3	1186	-2.8	0.0579
Temp Pel X TropPel	0.49	6.95	1186	0.07	1.0
Temp Pel X TropReef	-38.2	5.9	1186	-6.5	<0.0001
TempReef X TropDem	-23.3	5.1	1186	-4.6	0.0001
TempReef X TropPel	-2.5	4.6	1186	-0.54	0.9944
TempReef X TropReef	-41.2	2.8	1186	-14.9	<0.0001
TropDem X TropPel	20.8	6.2	1186	3.4	0.0107
TropDem X TropReef	-17.9	5	1186	-3.6	0.005
TropPel X TropReef	-38.7	4.5	1186	-8.5	<0.0001

Table 3.21 Summary statistics for the relationship between U_{crit} and muscle area (mm^2) for post-flexion larvae from six 'Environmental Habitats' (temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), at a common body size (16-22mmTL)

Model=lm(Muscle.area~Habitat, data)

	Est.	**Std. error**	**T**	**p**
Intercept	26.9	5.4	4.9	1.5e-06
HabitatTempPel	-3.8	6.1	-0.613	0.54
HabitatTempReef	-0.017	5.9	-0.003	0.998
HabitatTropDem	22.3	7.1	3.1	0.00199
HabitatTropPel	31.8	9.7	3.3	0.00116
HabitatTropReef	42.8	5.9	7.2	9.08e-12

$F_{5,209}$=46.58

Table 3.22 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in U_{crit} between post-flexion larvae from six 'Environmental Habitats'(temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), and muscle area (mm^2), at a common body length (16-22mm).

	ratio	**SE**	**df**	**t**	**p**
TempDem X TempPel	3.8	6.1	209	0.61	0.99
TempDem X TempReef	0.017	5.9	209	0.003	1.0
TempDem X TropDem	-22.3	7.1	209	-3.1	0.024
TempDem X TropPel	-31.8	9.7	209	-3.3	0.015
TempDem X TropReef	-42.8	5.9	209	-7.2	<0.0001
TempPel X TempReef	-3.7	3.8	209	-0.99	0.9211
TempPel X TropDem	-26.1	5.4	209	-4.8	<0.0001
Temp Pel X TropPel	-35.6	8.5	209	-4.2	0.0006
Temp Pel X TropReef	-46.6	3.7	209	-12.6	<0.0001
TempReef X TropDem	-22.3	5.2	209	-4.3	0.0004
TempReef X TropPel	-31.9	8.4	209	-3.8	0.0025
TempReef X TropReef	-42.8	3.4	209	-12.5	<0.0001
TropDem X TropPel	-9.5	9.2	209	-1.03	0.907
TropDem X TropReef	-20.5	5.2	209	-3.9	0.0015
TropPel X TropReef	-10.9	8.3	209	-1.3	<0.0001

Table 3.23 Summary statistics for the relationship between U_{crit} and caudal fin area (mm^2) for post-flexion larvae from six 'Environmental Habitats'(temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef)

Model=lm(Caudal.fin.area~Habitatdata)

	Est.	**Std. error**	**T**	**p**
Intercept	6.07	0.36	17.0	<2e-16
HabitatTempPel	-1.5	0.85	-1.7	0.083
HabitatTempReef	0.63	0.46	1.4	0.17
HabitatTropDem	5.7	3.09	1.8	0.067
HabitatTropPel	0.12	0.68	0.18	0.85
HabitatTropReef	9.2	0.61	15.1	<2e-16

$F_{5,822}$=58.55

Table 3.24 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in U_{crit} between post-flexion larvae from six 'Environmental Habitats'(temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), and caudal fin area (mm^2).

	ratio	**SE**	**Df**	**t**	**P**
TempDem X TempPel	1.5	0.85	822	1.7	0.506
TempDem X TempReef	-0.63	0.46	822	-1.4	0.738
TempDem X TropDem	-5.7	3.1	822	-1.8	0.442
TempDem X TropPel	-0.12	0.68	822	-0.18	1.0
TempDem X TropReef	-9.2	0.61	822	-15.1	<0.0001
TempPel X TempReef	-2.1	0.83	822	-2.56	0.108
TempPel X TropDem	-7.2	3.2	822	-2.3	0.211
Temp Pel X TropPel	-1.6	0.97	822	-1.7	0.554
Temp Pel X TropReef	-10.7	0.92	822	-11.6	<0.0001
TempReef X TropDem	-5.05	3.08	822	-1.6	0.574
TempReef X TropPel	0.51	0.64	822	0.79	0.97
TempReef X TropReef	-8.6	0.57	822	-15.1	<0.0001
TropDem X TropPel	5.6	3.1	822	1.8	0.48
TropDem X TropReef	-3.5	3.1	822	-1.1	0.868
TropPel X TropReef	-9.08	0.76	822	-11.9	<0.0001

Table 3.25 Summary statistics for the relationship between U_{crit} and caudal fin area (mm^2) for post-flexion larvae from six 'Environmental Habitats' (temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), at a common body size (16-22mmTL)

Model=lm(Caudal.fin.area~Habitat, data)

	Est.	**Std. error**	**T**	**p**
Intercept	6.9	1.1	6.06	6.31e-09
HabitatTempPel	-2.3	1.3	-1.8	0.073
HabitatTempReef	1.6	1.2	1.3	0.193
HabitatTropDem	8.7	1.5	5.8	1.97e-08
HabitatTropPel	8.5	2.03	4.2	4.28e-05
HabitatTropReef	12.3	1.2	9.8	<2e-16

$F_{5,209}$=89.07

Table 3.26 Pairwise comparisons, using a Tukey's post-hoc test, of the differences in U_{crit} between post-flexion larvae from six 'Environmental Habitats' (temperate demersal, temperate reef, tropical pelagic, tropical demersal, tropical reef), and caudal fin area (mm^2), at a common body length (16-22mm).

	Ratio	**SE**	**df**	**t**	**p**
TempDem X TempPel	2.3	1.3	209	1.8	0.467
TempDem X TempReef	-1.6	1.3	209	-1.3	0.781
TempDem X TropDem	-8.7	1.5	209	-5.8	<0.0001
TempDem X TropPel	-8.5	2.03	209	-4.2	0.0006
TempDem X TropReef	-12.3	1.2	209	-9.9	<0.0001
TempPel X TempReef	-3.9	0.8	209	-4.9	<0.0001
TempPel X TropDem	-11.1	1.1	209	-9.7	<0.0001
Temp Pel X TropPel	-10.8	1.8	209	-6.1	<0.0001
Temp Pel X TropReef	-14.6	0.78	209	-18.8	<0.0001
TempReef X TropDem	-7.1	1.1	209	-6.5	<0.0001
TempReef X TropPel	-6.8	1.8	209	-3.9	0.0018
TempReef X TropReef	-10.6	0.72	209	-14.8	<0.0001
TropDem X TropPel	0.26	1.9	209	0.13	1.0
TropDem X TropReef	-3.5	1.09	209	-3.3	0.016
TropPel X TropReef	-3.8	1.8	209	-2.2	0.26

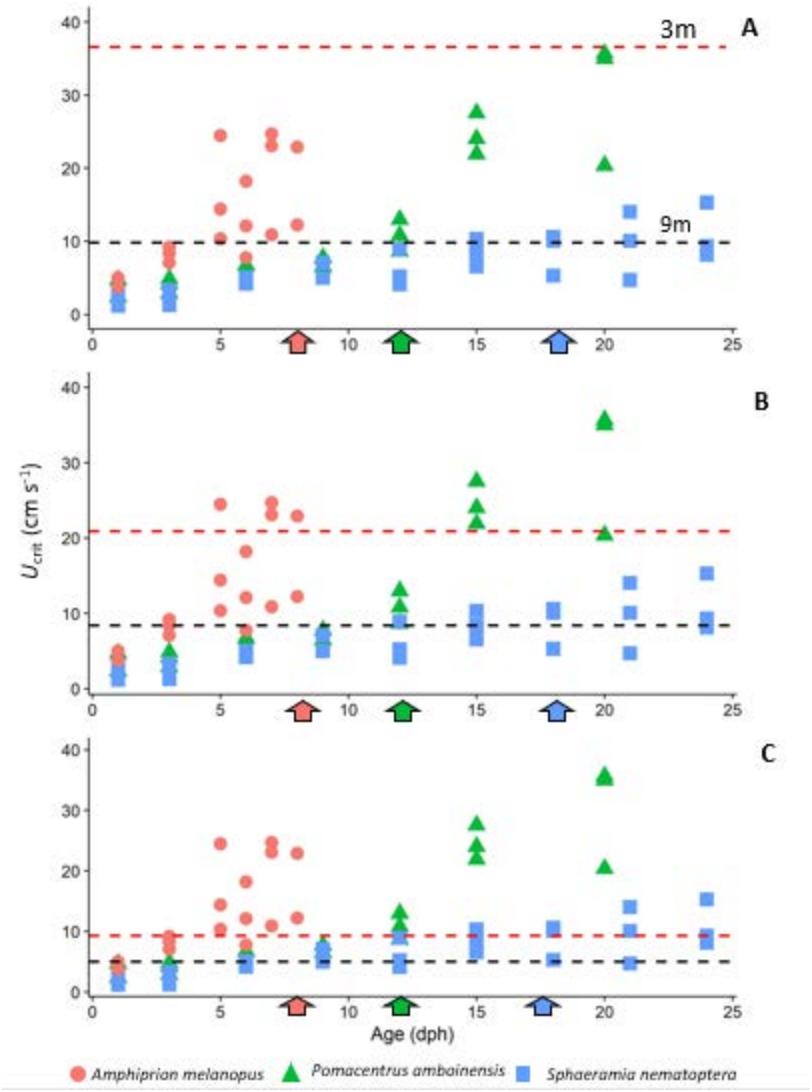

Figure 3.12 Critical swimming speed (U_{crit}; cm s^{-1}) over larval ontogeny (age; days post-hatch; dph) of laboratory-reared larvae of three coral reef fish species that hatch from demersal or brooded eggs (Cinnamon Anemonefish, *Amphiprion melanopus,* Pomacentridae ●, Ambon Damselfish, *Pomacentrus amboinensis,* Pomacentridae ▲ , and Pyjama Cardinalfish, *Sphaeramia nematoptera,* Apogonidae ■) (data adapted from Fisher et al., 2000) at different coral reef habitats on Lizard Island, Australia. **A.** exposed reef, **B.** oblique reef, and **C** sheltered reef (adapted from Johansen, 2014). Habitats are based on their level of exposure to south-easterly trade winds. Current data were measured using a custom-built flow meter, which was positioned at three replicate sites per reef habitat at depths of 3m (horizontal red dotted line indicates average speed at depth) and 9 m (horizontal black dotted line indicates average speed at depth) (Johansen, 2014). Upward arrows on the x axis match colours of their respective species and represents the age when larvae are capable of swimming in an inertial hydrodynamic regime (Re>1000). The last data point from each species represents the age at settlement.

Figure 3.12). *S. nematoptera* became capable of influential swimming at 18dph (~9.9 cm s^{-1}; 9.9mm TL; settlement at 23dph; Figure 3.12). On exposed reefs, none of these species at either the age when swimming becomes influential (*Re* >1000) or the age at settlement (last day of PLD) exhibited U_{crit} values that were faster than currents at 3m depth (Figure 3.12). In contrast, on sheltered reefs, all three species possessed U_{crit} values that were sufficient for them to swim against shallow and deep currents at the settlement stage (Figure 3.12). Settlement-stage larvae from all three species would be capable of swimming against deep currents on exposed, oblique, and sheltered reefs (Figure 3.12). In contrast, *A. melanopus* and *P. amboinensis* would be capable of swimming against shallow currents on oblique reefs upon settlement; whereas, *S. nematoptera* would not. (Figure 3.12).

3.4.4.2. Swimming performance of settlement-stage coral reef fishes versus Lizard Island current speeds

On average, across 10 families (30 species), settlement stage tropical coral reef fishes swim (i.e., U_{crit}), on average, 420 ± 54% above the minimum speed required to overcome the viscosity of seawater. This range of overcompensation spanned from 124% (*Apogon cyanosoma*; Apogonidae) to 1400% (*Pristotis obtusirostris*; Pomacentridae). Upon excluding species from families Apogonidae and Pomacanthidae, the average U_{crit} for all other species of fish examined would have been sufficient for the remaining fish species to swim against the fastest currents, which were the shallow currents on exposed reefs (Figure 3.13). *In situ* swimming speeds were sufficient for settlement-stage larvae of all coral reef species to surpass the deepest currents for exposed, oblique, and sheltered reefs and the shallowest currents on sheltered reefs, except in species from family Apogonidae (Figure 3.13). In contrast, U_{crit} swimming speeds of pelagic and demersal fishes would only suffice on sheltered reefs at the shallowest depths (Figure 3.13). Additionally, for demersal and pelagic fishes, *in situ* speeds would only suffice at deeper depths in any of the reef habitats and only at shallow depths on sheltered reefs (Figure 3.13).

3.5 Discussion

This chapter supports several decades of research showing that reef associated species are impressive swimmers. While tropical reef species have received much of the research attention, I show that temperate reef fish larvae are swimming to similar capacities as tropical pelagic and demersal species, despite the 10°C difference in water temperature. The interactions among biotic and abiotic factors that contribute to annual fluctuations in larval fish recruitment and dispersal are complex. One of these factors is the spatial scale in which fish larvae move, which suggests that swimming can play an important role in fish dispersal. Indeed, swimming performance is a critical trait that is universal among marine fishes, contributes to dispersal and recruitment processes, and is impacted by many biotic and

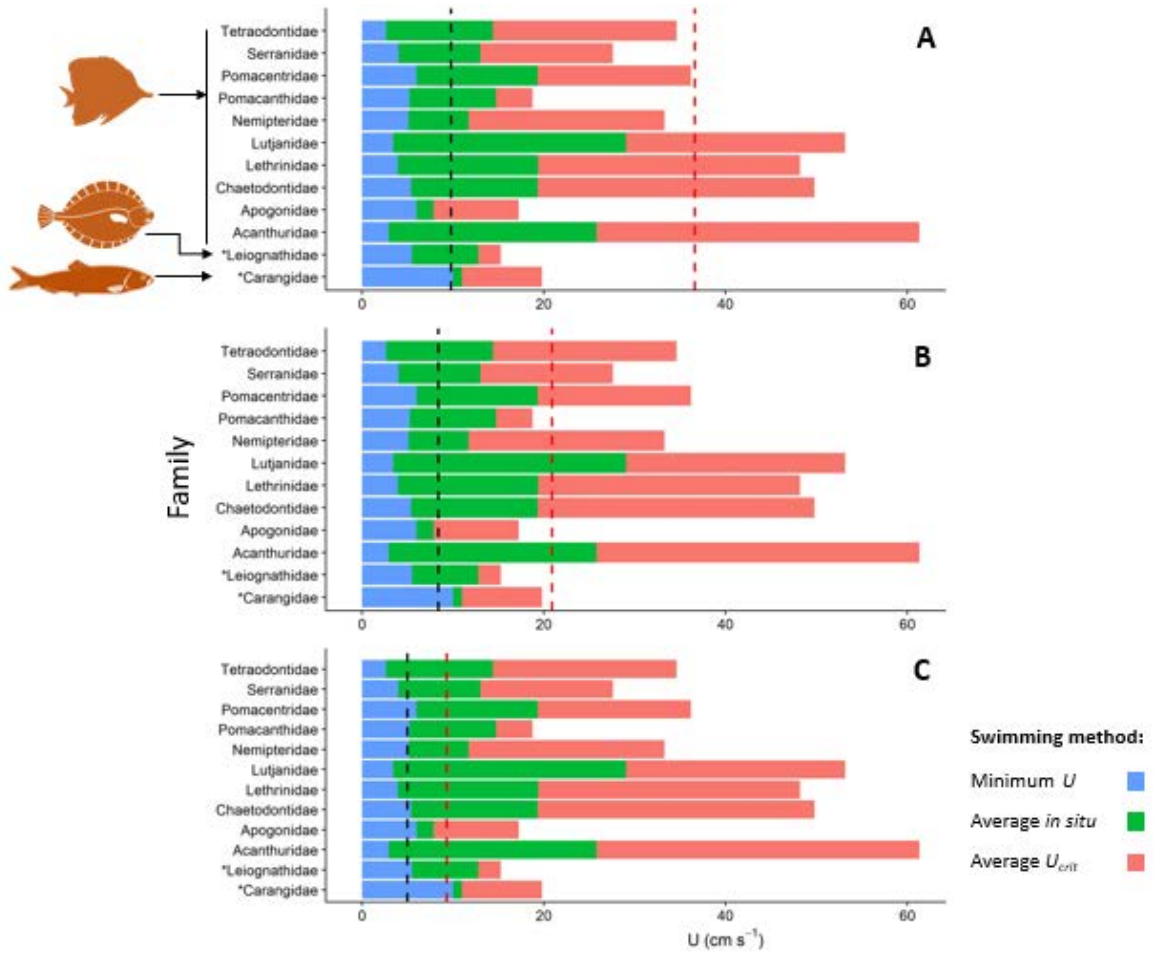

Figure 3.13 Comparison of different swimming performance (*U*; cm s^{-1}) metrics of settlement stage larvae across 12 different families of tropical, reef, and non-reef associated fishes. Average oceanic current velocity (cm s^{-1}) data from three different coral reef habitats: **A.** exposed, **B.** oblique, and **C.** sheltered reefs on Lizard Island (Australia). Habitats are based on their level of exposure to south-easterly trade winds. Current data were measured by a custom-built flow meter, which was positioned at three replicate sites per reef habitat at depths of 3m (vertical red line indicates average speed at depth) and 9 m (vertical black line indicates average speed at depth) (Johansen, 2014). 'Minimum *U*' (Min *U*;) represents the predicted minimum swimming speed required to overcome the viscosity of water (*U*, where *Re* =1000), '*in situ*' represents the average swimming speed under natural conditions ( ), and 'Average U_{crit}' is the mean swimming speed for larvae swimming at prolonged speeds ( ). Reef fish families are indexed by a butterflyfish image. The pelagic fish family is indexed by a herring symbol. The demersal fish family is indexed by a flatfish symbol. Symbols are indexed on 'Exposed' reef panels but are consistent with 'Oblique' and 'Sheltered' reefs. Swimming data were adapted from Fisher and Leis (2006).

abiotic factors (Downie et al., 2020). Although it had been well established that latitude and body size influence larval fish species' swimming speed (e.g., Hunt von Herbing, 2002), my analyses of the present larval fish swimming literature also suggest that the habitat with which adults associate (i.e., reef, demersal or pelagic) may have had an important role in selecting for swimming traits of larvae over evolutionary history. Post-flexion stage larvae of tropical and temperate reef fishes swim to a much higher capacity than non-reef counterparts, likely attributable to larger body sizes achieved by these groups at a similar stage of development and more robust body morphologies. This may support hypothesis 1 of my thesis, in that the physiological and morphological requirement of finding and selecting a patchy habitat (e.g., coral or rocky reefs) with different hydrodynamic conditions (e.g., strong nearshore currents, especially near the surface) on which to settle and transition to older life history stages – as opposed to living strictly in the pelagic realm or selecting other benthic habitats – may have underpinned selection for larger body sizes to produce higher performance among larval reef fishes. However, it is important to note that latitude is conflated with phylogeny, as percomorph species dominate warmer waters, and species of other non-percomorph orders (e.g., Clupeiformes, and Gadiformes) dominate cooler waters (Leis et al., 2013b). Therefore, it would be of interest to investigate more species from orders or families with wide latitudinal ranges. However, despite the obvious differences in temperatures between latitudes, it is important to note that my analyses found that: 1) at the same latitude (e.g., experiments were conducted at 26-30°C), tropical reef fish larvae swim to a higher capacity than tropical pelagic and demersal fish larvae, and 2) temperate reef fish larvae were able to swim to similar speeds as tropical demersal and tropical pelagic species, despite the 10°C difference in experimental water temperatures.

3.5.1. AIM I: development of swimming performance

Absolute swimming speed (cm s^{-1}) generally increases with body length (relative swimming speed, body lengths s^{-1}, levels off with increasing size in adults; Kolok, 1999) and is correlated with temperature (Moyano et al., 2016), growth rate (Billerbeck et al., 2001), and diet composition/ration size (Arnott et al., 2006). I found similar trends when comparing swimming speed (U_{crit} and *in situ*) with body length across 'Environmental Habitats'. Larvae of tropical species generally had greater increases in U_{crit} with increasing body length than temperate pelagic and temperate demersal species (Kingsolver & Huey, 2008). Interestingly, temperate reef fish larvae had the fastest increase in U_{crit} with increasing size, thus highlighting the complexities of modelling swimming performance in relation to ontogeny across latitude for marine teleost larvae, as many biotic and abiotic factors contribute and occasionally affect each other (Leis et al., 2013b). Several studies focusing on fishes (e.g., Atlantic cod *Gadus morhua* Sylvestre et al., 2007; Atlantic silverside *Menidia menidia* Atherinopsidae, Billerbeck et al., 2000, 2001) support counter-gradient variation, whereby some species inhabiting colder habitats have faster growth rates than species

inhabiting warmer habitats (reviewed by Kingsolver & Huey, 2008). These studies found that the caveat to faster growth in high latitude species included significant changes in other physiological properties, notably decreased swimming performance and increased standard metabolic rate and specific dynamic action (Billerbeck et al., 2000, 2001; Gregory & Wood, 1998; Kolok & Oris, 1995). This caveat may not hold true for temperate reef fish larvae during early larval stages in terms of swimming performance (see AIM II), but trade-offs with other physiological traits may exist.

During the larval phase for marine fishes swimming capabilities generally increase with length and girth (i.e., muscle mass) such that a specific level of performance can be accomplished to improve the chances of encountering prey items and achieve ontogenetic milestones that contribute to overall survival in their respective habitats. Additionally, the ability to swim faster and for longer periods may have a ripple effect on their dispersal potential, which is influenced further by environmental temperatures, resource abundance (Llopiz, 2013), and interspecific differences in muscle composition (e.g., proportion of red muscle and muscle mitochondria volume density; Johnston et al., 1998), active and standard metabolic rates (Peck & Moyano, 2016), food consumption rates, and morphology (e.g., body depth, finess ratio, and fin aspect ratio; Fisher & Hogan, 2007). How larval fishes metabolise energy over development and the extent to which energy is diverted to growth (i.e., organogenesis and hyperplasia and hypertrophy of muscle fibers), maintenance, and swimming performance at key developmental milestones (e.g., notochord flexion) are generally unknown across a wide range of species, but would be highly species-specific and likely driven by several biotic and abiotic factors (Wieser, 1995; Wieser & Kaufmann, 1998). However, even *in situ*, larvae of tropical reef fishes exhibit faster swimming speeds as they get larger, when compared to tropical pelagic and temperate fishes. The exact physiological mechanisms and environmental pressures underpinning developmental rates and dispersal potential among temperate reef fishes are also generally unknown, but clearly warrant further investigation to better understand how higher latitude species increase swimming performance with growth faster than lower latitude species.

3.5.2. AIM II. Comparing swimming performance of settlement-stage larvae

Nearly all teleost fishes are ectotherms, and therefore, metabolic rate increases with increasing water temperature, which has strong, positive correlations with increased muscle function and swimming speed (Hunt von Herbing, 2002). Therefore, it is important to mention early in the discussion of my results that water temperature is generally confounded with 'Environmental Habitat', which is an important factor to consider when comparing traits amongst these habitats. Temperate species were generally swum at temperatures below 15°C, and tropical species were swum between 26 and 30°C. Moreover, the differences between these groups with respect to swimming performance are apparent

(e.g., Orders Gadiformes, Clupeiformes, and Pleuronectiformes from cold waters compared to tropical species). Comparing among tropical species clearly shows that reef fishes swim to a higher capacity than pelagic and demersal species, despite the common temperature. Interestingly, some of the temperate species (i.e., temperate reef fishes) swum between 15 and 20°C exhibited swimming performance that was comparable to tropical species, despite the 10°C difference in water temperature. These species were from warm temperate regions, such as the Mediterranean Sea (e.g., Faillettaz et al., 2018) and south eastern coast of Australia (e.g., Clarke et al., 2005), regions that naturally experience wide fluctuations in annual temperatures. For example, surface temperatures in the Mediterranean Sea have high seasonal fluctuations (9.0-17.7°C in winter and 20.8-28.3°C in summer; Shaltout & Omstedt, 2014). In contrast, tropical and cold temperate regions do not experience such dramatic shifts in annual sea surface temperatures (see references discussed in Methods section in Illing et al., 2020). Additionally, Downie et al., (2020) found no overall change in swimming performance in response to thermal stress of species from warm-temperate regions. Tolerance to a wide thermal regime may attribute to the high swimming capacity of the larvae of temperate reef fishes, especially if the upper thermal limits of their habitat are similar to temperatures experienced by tropical species. It may be of interest to swim temperate fishes that naturally experience wide seasonal variations in temperature across this thermal range. Regardless, across several metrics of performance, reef-associated species generally exhibit a stronger swimming performance than fishes from other groups.

In absolute terms (e.g., U_{crit} in cm s^{-1}), tropical reef fishes are capable of attaining higher U_{crit} and *in situ* speeds than temperate and tropical non-reef fishes (Clark et al., 2005; Fisher et al., 2005; Leis et al., 2006; Leis, 2010). While casually referred to as 'athletes', this high-calibre performance in tropical reef fishes was widely considered to be driven by latitude (Leis & McCormick, 2002; Leis, 2007; Downie et al., 2020). These results are complimented by the fast speeds that settlement-stage tropical reef fishes exhibit *in situ*, especially when compared to temperate and tropical pelagic larvae, along with a suite of complex behaviours and sensory abilities exhibited by tropical reef fish larvae (e.g., swimming depth and orientation) that are associated with their ability to find and settle onto a reef habitat (Leis et al., 1996; Leis & Carson-Ewart, 1999; Leis et al., 2009). Several studies have found interspecific differences in red muscle composition, oxygen uptake rates, and muscle mitochondria volume density between fishes at different latitudes and lifestyles, with fishes from low latitudes having higher respiration rates and aerobic properties in their muscle tissues than fishes living in colder habitats (Johnston et al., 1998; Killen et al., 2010). This may be further complimented by body size, in which propulsive area of swimming muscles and muscle mass increases with size (Fisher & Hogan, 2007). However, it is interesting that post-flexion temperate reef fishes are capable of swimming to a similar capacity as tropical pelagic and demersal species (Leis et al., 2012; Faillettaz et al., 2018) , possibly attributed to the high growth rates during early

larval ontogeny (see Aim I), as well as their wide thermal tolerance ranges. This is accentuated when body size is taken into account, and this relationship holds true in the endurance swimming data as well.

Larvae of tropical reef fishes were previously noted for having high dispersal potential based on long distances (>30km) swum during endurance experiments (Stobutzki & Bellwood, 1997; Fisher & Bellwood, 2001) . However, fishes from the Fisher & Bellwood, (2001) study were fed throughout endurance tests; whereas, food is generally withheld for 24 h prior to most endurance tests (Downie et al., 2020). My analyses excluded fishes that were fed during endurance tests, and as such, I found that temperate reef fishes were capable of similar endurance swimming distances as tropical fishes (e.g., Dudley et al*.,* 2000; Clark et al., 2005; Baptista et al., 2019). Post-flexion stage fishes, regardless of latitude, likely need strong endurance capacity to find patchily distributed resources in the open ocean, especially at low latitudes where these resources are more limited (Llopiz, 2013). Comparing energy reserves and metabolic rates between tropical and temperate groups within the context of endurance swimming may reveal more to this interesting contrast between endurance capacity. This endurance capacity is especially important when combined with finding from Nilsson et al., (2007a) whom found that tropical reef fish larvae have the highest oxygen uptake rates of any other teleost fish during activity, suggesting reef fish larvae are energy efficient swimmers. Indeed, while reef fishes are among the fastest swimmers and capable of long-distance swimming, it is important to note the influence that body size has on such performance metrics.

There has been focus on the relationship between body size and swimming performance when making interspecific and intraspecific comparisons among fishes across ontogeny (Kolok, 1999; Fisher et al., 2005; Fisher & Hogan, 2007; Fisher & Leis, 2010). Among settlement stage larvae of 100 coral reef species from Australia and the Caribbean, ~70% of the variation in swimming speed (U_{crit}) was accounted for by body length and other morphological metrics, such as caudal peduncle depth and fin aspect ratio (Fisher & Hogan, 2007). Similarly, my models suggest that body length has a large influence on swimming performance, as temperate reef fishes and tropical pelagic, reef and demersal fishes of the same size range (16-22mm SL) do not differ significantly in swimming performance, and the larger sizes of larval reef fishes in general – both temperate and tropical – may be driving this performance metric, particularly among tropical species (see Figures 1 and 2 in Leis, 2010). Given the phylogenetic overlap, in the form of a large bias toward percomorph species between tropical and temperate reef fish larvae, it is possible that these groups of fishes have inherited similar properties in their muscle tissue and metabolic rates at a similar size. However, very little is known about properties of muscles (e.g., mitochondrial volume densities) from tropical reef fishes, and I suggest that this is an area of particular interest for future studies. Furthermore, as evidenced by my analyses on changes in swimming speed with body

length (Aim I), interspecific growth rates may also contribute to the expression of swimming genotypes among groups of fishes (Kolok & Oris, 1995; Billerbeck et al., 2000, 2001). However, I found *in situ* swimming speed was still highest among larval tropical reef fishes, when compared to larvae of tropical pelagic and demersal fishes, even when accounting for body size. While few studies have investigated the relationship between *in situ* swimming and laboratory estimates (e.g., U_{crit}), there appears to be no fixed relationship, and the relationship appears highly variable among species (Leis, 2020). Taken together, larvae of reef fishes from both high (i.e., temperate) and low (i.e., tropical) latitudes are capable of fast swimming speeds at settlement when compared to their non-reef counterparts. This may be attributed to the robust body morphologies that the majority of reef fishes have –at least in the species studied thus far- that may be specialized for swimming.

Fishes exhibit a wide diversity of body shapes, from dorsoventrally flattened (e.g., Order Pleuronectiformes) to streamlined (e.g., Order Scombriformes) (Walker et al., 2013). Body morphology and swimming performance are positively correlated, as more streamlined body morphologies reduce drag and enhance swimming performance (Fisher & Hogan, 2007). Additionally, the morphology of the caudal fin (e.g., depth and area) provides thrust for forward momentum (Webb, 1984), and unusually designed pectoral fins (e.g., members of the Family Labridae) enable adult tropical reef fishes to swim against strong reef currents (Fulton & Bellwood, 2004; Fulton et al., 2013). Near the end of their larval phase, the larvae of many fishes have already attained their adult morphologies in shape, despite their small sizes, and are generally swimming in inertial hydrodynamic regimes (e.g., Re >1000), making it therefore reasonable to use body shape as an important factor influencing swimming during early life stages (e.g., Fisher & Hogan, 2007). I found that body shape has an important influence on swimming performance among larval fishes across 'Environmental Habitats'. The best performers were generally round or streamlined in shape (body length:body depth ratio ranging 1 to 3), which were the morphologies most frequently exhibited by reef fishes. In contrast, temperate pelagic fishes were generally more 'ribbon shaped' or slender in shape (e.g., Order Clupeiformes) and had weaker swimming performance. It is interesting that the rounded shaped/robust fishes had high swimming performance, as such body shapes would expect to create more drag. Swimming performance was found to be greatest among adult tropical reef fishes with high (e.g., more streamlined) finess ratios (relationship between body depth, length, and width) versus low finess ratios (circular shaped) (Walker et al., 2013). This suggests that the physiological requirements to swim toward a reef during early life may be different than the requirements and demands to stay on a reef as an adult (Fulton & Bellwood, 2004; Fulton et al., 2005; Walker et al., 2013). However, I found that the higher swimming performance of fishes with more robust body shapes may be because these morphologies had greater muscle areas than thinner body morphologies. Therefore, growing larger to increase muscle mass may be a strategy for body

morphologies that are more-susceptible to drag to swim faster against ocean currents. This also implies that physiological metrics that are involved in muscle function during exercise, such as oxygen uptake rates, mitochondria volume densities of red muscle, and enzyme activities that are involved in cellular respiration, may be important predictors of swimming performance among settlement-stage larvae. Unfortunately, these metrics and the muscle development of reef fishes are unknown, and there is a general lack of knowledge with respect to aerobic properties of swimming muscles from tropical species, when compared to temperate, commercially-important species (e.g., Orders Clupeiformes, Gadiformes, Pleuronectiformes, etc.), and this would be an area of particular interest for future studies. Growing larger to accommodate larger blocks of muscle, especially for robust species vulnerable to drag, allows larvae to swim faster, which may be valuable and selected for to enhance recruitment for reef fishes and subsequent performance during post-settlement processes.

3.5.3. AIM III. Case study: swimming performance over ontogeny versus Lizard Island current velocities

In the field of exercise physiology, the aim is to better understand how and why animals move to the extents that they do (Bennett, 1991; Irschick, 2002; Irschick & Higham, 2016). Putting performance into an environmental context requires both natural and laboratory measures of a trait (Irschick, 2003). Fortunately, for tropical fishes, laboratory estimates of maximum, prolonged swimming speeds (U_{crit}) and *in situ* observations are available, and my case-study approach incorporates both estimates. The large sizes of some (i.e., the ones in our analyses) tropical coral reef fishes before they leave the pelagic environment likely contribute to the high U_{crit} and *in situ* speeds. However, upon hatching, larval marine fishes are tiny, and their swimming speeds are not sufficient for them to remain on reefs (Leis & McCormick, 2002; Leis, 2006). The few species in which a pelagic stage is lacking are mouth brooders (e.g., Apogonids) or provide parental care, with parents keeping well-developed hatchlings sheltered in enclaves, caves, and among coral (e.g., spiny chromis; *Acanthochromis polyacanthus,* Pomacentridae). While some studies have attempted to explain/quantify the persistence of a pelagic larval duration among reef fishes (e.g., competition with adults for resources, predators, etc.; Bonhomme & Planes, 2000), the inability to remain on reefs due to underdeveloped swimming performance structures (e.g., fins and muscles) may provide a more straightforward answer. Most reef fishes have pelagic larval durations that span several weeks to months (Leis, 2006). Once these larvae are capable of swimming (i.e., inertial *Re*), they have possibly been moving away from the reef for several days/weeks (Leis, 2006). Indeed, the need to influence control of their movement in the pelagic environment may allow them to seek out a specific habitat – either natal (e.g., Jones et al*.,* 1999, 2005) or new (e.g., Harrison et al., 2012) – which may be an important driver influencing the fast swimming performance among reef-associated species.

High swimming performance among reef fish larvae may provide a good example of how an animal is truly a product of its environment. Several studies have shown intraspecific differences in swimming performance between isolated populations of fishes exposed to different riverine or oceanic flows, with individuals from higher flow habitats exhibiting better swimming performance (Taylor & McPhail, 1986; Taylor & Foote, 1991; Nelson et al., 2003). Therefore, over evolutionary history, the pressure to find a specific, patchily distributed reef habitat and outswim distinct oceanic and reef currents may have selected for the high performance of larval reef fishes, regardless of latitude. This may be why tropical and temperate larval reef fishes grow to larger sizes than larvae of pelagic and demersal fishes at similar latitudes. The U_{crit} and *in situ* swimming speeds of most tropical larval reef fishes are faster than the high reef currents at different depths and locations on Lizard Island in the northern Great Barrier Reef, which indicates, therefore, performing at a bare minimum (e.g., Re=1000) would not suffice against these currents. Across 57 species of tropical fish larvae surveyed via plankton tows at different locations around Lizard Island, 50% of post-notochord flexion larvae were found at depths of 6 m during the day, and 40% were found near the surface at night (Leis, 1986). Surface currents in these areas ranged from 3.5-37.5 cm s^{-1}, and currents at the deeper depths (7.5m) ranged from 0.9-25.5 cm s^{-1}, depending on location (Leis, 1986). Some of these current speeds would not be surpassed by *in situ* swimming alone, but U_{crit} speeds would suffice. These currents are relatively high, and reef fish larvae captured by Leis (1986) and during other studies (e.g., Leis & Goldman, 1984; Leis & Goldman, 1987) typically occur within the depths indicated by my case-study. These reef currents, particularly on the exposed reefs, are too strong for non-reef tropical larvae. While simplistic in nature, comparing natural and laboratory measurements of swimming speeds with oceanographic data may further our understanding as to the degree by which ambient currents have influenced swimming performance in reef fishes (see Fisher, 2005 as an example).

Given the simplistic approach to this case study, however, I do need to be cautious in my interpretation. Understanding the temporal (e.g., diurnal and seasonal patterns) variations in vertical distributions patterns of ichthyoplankton is complex and has been an area of particular interest since the 1980s for tropical species (possibly even longer in temperate waters), with the general consensus suggesting that distributions vary considerably with location, taxa, thermocline, hydrology, and prey distribution patterns (Leis, 1991; Olivar & Sabatés, 1997; Rodríguez et al., 2006; Höffle et al., 2013). For example, in the North Sea, vertical distributions of larvae are taxon-specific, with Gadiformes selecting depths below 40m during the day, and Pleuronectiformes selecting depths ranging 20-90m regardless of time of day (Höffle et al., 2013). In contrast, in the Mediterranean, while some species were found consistently near the surface (e.g., *Boops boops* Sparidae, and *Diplodus sargus* Sparidae), despite seasonal changes in hydrology (Olivar & Sabatés, 1997), and species found off the coast of Africa were consistently found at intermediate depths (Rodríguez et al., 2006). The vertical distributions of tropical

reef fishes are variable, both temporally and spatially; yet, many species are consistently found near the surface at night (e.g., Leis, 1986) and some during the day (Leis, 1991). The role of the case study was to provide some environmental context as to why tropical reef fishes swim to such a high capacity. Ongoing research is examining the role of fish behaviour in relation to the hydrodynamic environment that tropical reef fishes experience as they approach a reef and how it contributes to dispersal, but evidence suggesting that tropical reef fishes display a high level of self-recruitment implies that swimming against currents likely plays a critical role (Jones et al., 1999; Christie et al., 2010; Saenz-Agudelo et al., 2011). The level of self-recruitment is generally unknown for temperate reef fishes, but is an area of particular interest for future studies. In contrast, temperate pelagic and demersal fishes like herring, cod, and flatfishes rely more heavily on hydrodynamic processes (e.g., current stratification, wind) to bring larvae to nursery grounds, which may be some distance from spawning grounds, and do not actively move horizontally against currents (i.e., they act more like plankton, making vertical movements) until much later in their development (van der Molen et al., 2007; Dickey-Collas et al., 2009; Tanner et al., 2017; Lacroix et al., 2018). While I only used one location on the Great Barrier Reef for my case study, the hydrodynamic and biological processes that occur on Lizard Island are representative of recruitment conditions encountered by settling reef fishes regardless of location. However, the spatial scale over which dispersal, recruitment, and connectivity act is a critically important factor. Many studies investigating the self-recruitment and dispersal of tropical reef fish subpopulations occur among island reefs (e.g., Keppel Islands, Harrison et al 2012; Lizard Island, Jones et al 1999; Kimbe Island, Almany et al., 2007); however, some studies use larger spatial scales for better management practises for marine protected areas (MPAs). For example, across a 10 000 km^2 sampling range, clownfish (*Amphiprion percula,* Pomacentridae) larvae consistently dispersed 10-15km among local reserves over a two-year period (20% self-recruitment rate), and butterflyfish (*Chaetodon vegabundus,* Chaetodontidae) dispersed up to 64 km to local reserves (Almany et al., 2017). This shows that tropical reef fishes are targeting specific habitats within a specific spatial scale, and this may be consistent, temporally. The integration of species-specific larval behaviour into hydrodynamic models over appropriate spatial scales (e.g., within the maximum dispersal distances of larvae) will benefit the design of MPAs, and this is an area of ongoing investigation for both tropical and temperate regions. Future work on energetics, including oxygen uptake rates, muscle tissue development, etc., that support swimming behaviour will assist in better understanding how active behaviours contribute to the dispersal processes of marine fishes.

3.6. Conclusions

Swimming performance in marine teleost larvae is impacted by numerous biotic and abiotic factors, including latitude, habitat association, morphology, and body size, to name a few. The

interactions among these factors are complex, and more information regarding species-specific metabolic rates, composition of swimming muscles, feeding rates, and energy utilisation is needed in order to further differentiate swimming abilities. However, based on the available data, reef-associated percomorph fishes from high and low latitudes show impressive swimming capabilities when compared to pelagic and demersal counterparts. Following hypothesis 1 from my thesis, I conclude this is may be due to the need to grow larger to swim against oceanic and reef currents in order to select for new or natal reef habitats on which to settle (e.g., Jones et al., 1999, 2005). The high performance of temperate reef fishes when compared to high and low latitude non-reef fishes warrants further investigation into the anatomical and physiological mechanisms underpinning such performance. Although it is surprising that temperate reef fishes have the greatest increase in U_{crit} with body length, this highlights that the way in which performance scales with ontogeny varies across taxa and latitude, and that the way in which fishes metabolise and utilise energy may be different among taxonomic and ecological groups. It is important to note, however, that there is a profound sampling bias in the larval fish swimming literature toward percomorpha fishes (Leis et al., 2013b). While percomorph fishes represent the higher performing taxa, particularly on tropical reef habitats, this finding may be confounded by the bias that percomorph fishes constitute a greater proportion of low latitude coastal fish communities than high latitude communities. In other words, apparent influences of latitude are likely due to the species involved. Therefore, it would be invaluable for future research to investigate swimming performance of tropical species from taxonomic groups primarily found in high latitude areas (e.g., Pleuronectiformes and Clupeiformes) and vice versa. More information on the swimming performance of late-stage larvae from high latitudes would help minimise this bias in the data. Additionally, tropical and temperate reefs are heavily populated by cryptobenthic species (e.g., Gobiidae and Blenniidae; e.g., Majoris et al., 2019) whose larvae are not well represented in the larval fish swimming literature, and may not readily swim in flume chambers. The absence of data on the swimming performance of these fishes during their pelagic stages may have influenced our findings, and therefore it is important to emphasise that our findings are based on the available larval fish swimming literature. The species for which data on larval swimming are available are neither a representative nor a random sample of the fish communities from various latitudes or habitats. Therefore, I do note that caution should be taken when making gross generalisations from my results. Regardless, as more information becomes available on a wider range of species, my hypothesis that reef association influences swimming performance among fish larvae should still be considered. Ultimately, by placing swimming performance of marine teleost fish larvae within a phylogenetic framework and accounting for key biological factors impacting performance, we can further our understanding of swimming capacity in different groups of fishes. This, in turn, may provide a broader understanding of

the ecological and biological drivers of fish life history, including connectivity, mortality, feeding, dispersal, and recruitment.

This chapter supports the hypothesis that habitat association may play an integral role in the swimming performance of fish larvae. Specifically for reef fishes, the need to find a coastal reef habitat with distinct flows at the end of their larval stage may be why these larvae swim so fast. To support this they attain larger sizes than pelagic and non-reef demersal species, suggesting they have high rates of muscle tissue synthesis. The next chapter of my thesis will investigate how reef fish larvae develop muscle tissue; the machinery supporting athletic performance.

Chapter 4: Muscle tissue development of a coral reef fish species during embryonic and larval stages

4.1 Introduction

Swimming performance in fishes is largely powered by skeletal muscles, which comprise >60% of adult body mass for pelagic species (Johnston et al., 2011). Given the integral role swimming has for the survival of fishes, such as navigating through their environment, predator-prey interactions, reproduction and dispersal/migrations, it is therefore no surprise that muscle growth and development plays an integral role in fish ontogeny. Unlike mammals where the number of muscle fibers is set and post-natal muscle growth is achieved by hypertrophy of fibers alone (increasing fiber size), fish are constantly recruiting new muscle fibers until they reach their maximum size, because muscles reach a maximum diameter (50-200μm) (Johnston, 1999). Therefore increasing girth for larger species is achieved by hypertrophy and hyperplasia (i.e., increasing the number of muscle fibers), whereas hypertrophy alone is generally exhibited by smaller species (Brooks et al., 1995). As such, muscle is considered to be the fastest growing tissue, especially in early life, with some species such as the common carp (*Cyprinus carpio)* averaging 28% daily increases in muscle mass between the ages of 4-18dph (Alami-Durante 1990). The relationship between swimming and survival in early life is directly related to muscle development, and as such, much focus has been placed on understanding how teleosts develop muscle tissue.

While patterns of myogenesis (muscle development) do vary across vertebrate lineages, the basic principles of how muscle cells form are retained. However, it should be mentioned early that muscle development has primarily been investigated in zebrafishes (e.g., *Danio rerio*) and rainbow trout (*Onchorynchus mykiss*), as well as a few other species including Atlantic herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*). Additionally, the rate of myogenesis and inter and intraspecific differences in myogenesis is influenced by external stimuli such as temperature (Johnston, 1999; Hall & Johnston, 2003) . Regardless, common to all vertebrates is the designation of embryonic stem cells into myogenic cells (myoblasts) which continuously replicate, then abruptly leave the cell cycle (i.e., stop replicating), and differentiate into muscle cells by fusing myoblasts together to form multi-nucleated cells (somites) (Johnston, 2006). New muscle cells form on top of pre-existing muscle fibers, and are derived from satellite cells, which are sub-populations of cells that develop during embryogenesis (Johnston et al., 2011). These somites are pre-designated to develop into either red and white muscle (the expression of these muscle types becomes realized later in ontogeny, see below) or pioneer muscles (form myofibrils which allow muscle fibers to contract) (Johnston, 1999, Johnston et al., 2011). This process is under tight

genetic and hormonal control, and is initiated during the embryo phase and continues once the larva hatches from its egg.

Upon hatch, the muscles of fishes are not differentiated as either red or white muscle yet, and as such swimming behavior is highly limited at this stage. Instead established muscle blocks (composed of a superficial layer and deep fibers) that develop during embryogenesis act as a hydrostatic skeleton supporting the body until adult muscle fibers develop (**chapter 2**, Downie et al., 2020). Additionally, gills are not formed at hatch and oxygen is diffused into the body through the skin by a thin layer of superficial muscle tissue (called 'red layer'; El-Fiky et al., 1987). Different swimming behaviors and speeds are supported by the two distinct muscle types. Red or slow fibers are located on the periphery of the body cavity (form an inward V-shape), contain a high concentration of mitochondria, and are used for slow steady swimming (Johnston et al., 2011). White fibers constitute most of the muscle mass of a fish and contain few mitochondria and are anaerobically powered for burst speed (Johnston et al., 2011). At hatch, both the red layer and the deep fibers have high amounts of aerobic enzymes (e.g., citrate synthase and cytochrome oxidase), and high amounts of mitochondria, and these fibers are believed to be used for respiration (El-Fiky et al., 1987). The red layer becomes adult red muscle, and the deep fibers become adult white muscle and in effect, loses its aerobic properties (El-Fiky et al., 1987). The switch from larval to adult fibers coincide with particular ontogenetic milestones as larvae metamorphose into juveniles, primarily gill development, blood circulation and the onset of exogenous feed after the absorption of the yolk sac (El-Fiky et al., 1987; Talesara & Urfi, 1987; Vieira & Johnston, 1992; Downie et al., 2020). During this transition, swimming performance generally increases significantly (**chapter 2**, Downie et al 2020). As mentioned previously, much of this work on larval muscle (and gill) development has focused on a narrow range of species, most of which live in temperate waters, and most of these species are poorly developed at hatch. What about tropical species, such as anemonefishes, that hatch as well-developed larvae?

Several studies have investigated how anemonefishes and clownfishes develop throughout embryonic, larval, and juvenile stages, noting the time points when organ systems begin to develop. These fishes are direct-developers, meaning adults place a considerable amount of energy into producing fewer eggs adhered to the benthos that hatch into better-developed larvae than broadcast spawning fishes (i.e., fishes that release sperm and eggs into the water column, producing thousands to millions of poorly developed larvae). For zebrafish, herring, cod, and other studied groups, adult muscle fibers form in conjunction when the respiratory and circulatory systems are functional during late larval/early juvenile phases. However, several studies have found that clownfishes and anemonefishes have these structures in place upon hatch. Gills and the heart were formed nearly 4 days after fertilization (dpf) for spinecheek

anemonefish (*Premnas biaculeatus*; Madhu et al., 2012) and 5 dpf for false-percula clownfish (*Amphiprion ocellaris*; Yasir & Qin, 2007). Gills are used for oxygen uptake at hatch by cinnamon anemonefish (and presumably other anemonefishes and clownfishes), as exposure to phenylhydrazine, which induces haemolytic anemia, thus effectively destroying the function of haemoglobin for binding and releasing oxygen, resulted in death for newly hatched cinnamon anemonefishes (*Amphiprion melanopus*; Prescott et al., in revision). Additionally, blood is already circulating throughout the body during the embryo stage: 5dpf for orange clownfish (*Amphiprion percula;* Dhaneesh et al., 2009 and *A. ocellaris*; Yasir & Quin, 2007) and 2.5 dpf for *P. biaculeatus* (Madhu et al., 2012). Moreover, muscle blocks are visible during the embryo stage; yet, it has not been determined if these blocks have differentiated into adult muscle fibers (Yasir & Quin, 2007; Dhaneesh et al., 2009; Madhu et al., 2012; Gunasekaran et al., 2017). Since the development of gills occurs prior to hatch, does that suggest that muscles are fully differentiated into red and white fibers (i.e., adult fibers) as well?

Given that anemonefishes seemingly break the mold of the typical fish development plan and hatch with a well-developed cardio-respiratory system, is it possible that they also have unique patterns in muscle tissue development? Since gills are formed prior to and functional at hatch (i.e., anemonefishes have functioning gills at hatch), are adult red and white muscle fibers also differentiated at this stage? This suggests that anemonefishes and predictably other direct-developing fishes have better swimming capabilities at hatch, providing these fish with an advantage when it comes to finding food and navigating through the water at an early stage of development when compared to other fishes that develop these structures later in ontogeny. Therefore, the aim of this chapter was to investigate the development of muscle tissue from the point of fertilization until settlement as a juvenile of a direct-developing anemonefish (*Amphiprion melanopus*). Using histochemical techniques, I stained tissue for red and white adult muscle, to determine at what point in development adult fibers are formed. This will allow me to test **hypothesis 2** of this thesis. Because gills and adult muscles generally form in conjunction with one another in other teleost groups, the presence of functioning gills at hatch for anemonefishes suggests adult muscle fibers are also formed. Additionally, I also predict that, when compared to other species, anemonefishes have faster rates of muscle tissue synthesis, likely due to differences in water temperature. Overall, investigating muscle tissue development of direct-developing fishes, like anemonefishes, would provide a new perspective on the development of teleost swimming muscles and may provide evolutionary context to their high swimming capacity later in life.

4.2 Methods

All housing of adult breeding pairs, rearing of larvae and experimentation occurred at James Cook University campus at the Marine and Aquaculture Research Facilities Unit (MARFU). All

husbandry and experimentation methods were approved by James Cook University's animal ethics committee (ethics number A2425)

4.2.1 Husbandry of study species

Adult breeding pairs of the cinnamon anemonefish (*Amphiprion melanopus*) have been established at MARFU since 2015 but were originally captured on the Great Barrier Reef by commercial divers (Cairns Marine). For this study, adult pairs were maintained in 60L flow-through outdoor aquaria at MARFU. Natural summertime water conditions were maintained throughout the duration of the experiment (temperature = 28°C, salinity = 33ppt), and fishes experienced natural photoperiods. Adults were fed twice daily using pellet food (NRD G12 Inve Aquaculture Inc., Salt Lake City, USA). Within each tank there was half a terra-cotta pot for shelter and a place for adults to lay their eggs. Tanks were cleaned weekly to maintain good water quality (ammonium levels kept below 0.04ppm, and nitrate levels were kept within 10-40ppm).

Adults generally lay eggs every two weeks, and eggs generally hatch 7-8 days later. On the day prior to predicted hatching, the terra-cotta pot was removed from the adult tank and promptly replaced with a blank pot and then transported in water to a 100L flow-through (water parameters: temperature = 28°C, salinity = 33ppt) larval rearing tank in a separate indoor room. A summer photoperiod 13h light to 11h dark was maintained in the larval rearing room during the experimentation period. An air stone was placed under the eggs to simulate parents aerating the eggs, and promote hatching. Upon day at hatching to 5 days post hatch (0-5dph), larvae were fed rotifers (*Brachionus* sp.) at a concentration of 20 individuals ml^{-1}. Additionally, from hatch to 3 dph, 3ml of algal paste (*Nannochloropsis* sp.) was added to the tanks to feed rotifers and provide shelter to the larvae from light-stress. From 3dph-9dph, larvae were fed freshly hatched *Artemia* sp. naupli *ad libtum*. During feeding, water was switched off for 1 h to prevent food and algae from being removed from the tank, allowing larvae to have adequate time to feed. Water was switched back on to flush the system and maintain water quality.

4.2.2 Histological analysis of tissue

4.2.2.1 Sample preparation and tissue freezing

Muscle tissue samples were taken daily, from the time eggs were laid, across the larval stage, until settlement (~17 days total). For reference, the anemonefish has a 7-8 day embryonic period and a 9-day larval period. Numerous egg clutches were required to achieve the proper sample size per developmental day (n=10 eggs per larvae per day; total of 18 developmental days). To prevent any parental effects, I used eggs and offspring from the same anemonefish breeding pair. Additionally, I

maintained all larvae in the same rearing tank to reduce tank effects, and I made the effort to feed the larvae the same amount of food so there were no differences in growth between clutches.

Upon the start of the embryonic period, I gently removed 10 eggs from the surface of the terra-cotta pot with tweezers. The eggs were then placed into a small beaker containing water from the parents' aquarium. I did this daily throughout the embryonic period. I then removed 10 larvae, daily, from the larval rearing tank from hatch (0dph) until the day of settlement (9dph). Eggs and larvae were transported to a biochemistry lab (JCU campus; building 19 room 118) where muscle preparation took place. In preparation for the incoming samples, a small dewar was filled with liquid nitrogen. A metal beaker filled with Isopentane (Sigma, Germany) was placed in the dewar and allowed to cool down to -80°C. Eggs and larvae were euthanized in an ice bath and subsequently weighed and measured (standard length). Larvae were decapitated (axial muscles are visible), and their caudal fin was removed because it is easier to keep the samples standing upright during snap-freezing process. This was difficult to do with eggs due to their egg cases, so the entire egg was frozen. Then, an aliquot, approximately 10mm in diameter, of Optimal Cutting Temperature (OCT; Tissue Tek) was place on a metal chuck. Each individual sample was placed with the anterior end facing upright within the OCT. More OCT was added if the sample was not covered. The chuck was then carefully lowered into the isopentane, thus snap freezing the tissue. Samples were stored in individual vials at -80°C until further processing.

4.2.2.2 Histochemistry staining of muscle fibers

Prior to sectioning and staining the succinate dehydrogenase (SDH) working solution was prepared fresh each day. Succinate dehydrogenase stains highly oxidative tissues (e.g., red muscle) a distinct blue-purple colour. First, a 0.2M Tris buffer (pH=7.4) stock solution was made by adding 250ml Tris stock (6.05g Tris (Hydroxymethyl) Methylamine [Sigma Chemicals, Germany] and 250ml distilled water) and 207ml of HCl stock solution (4.25ml concentrated HCl to 250ml of distilled water). The SDH working solution was made by mixing 2.4g sodium succinate (Sigma chemicals, Germany), 0.12g nitrotetrazolium blue (Sigma Chemicals, Germany), and 0.006g phenazine methosulphate (Sigma Chemicals, Germany) to 240ml of Tris buffer. This was stored at 5°C until use.

Snap frozen samples were cut into 8µm sections using a cryostat (Leica CM1860 UV cryostat, Leica biosystems, Nussloch, Germany). Sections were placed in groups on a SuperFrost Plus slide (Thermo Scientific). For each individual fish, a small group of sections were first stained with Eosin and Hematoxylin. This allowed me to be confirm that the sections were of good quality (e.g., no lesions, ice crystal artefacts) and contained axial muscle. Once this was confirmed, several sections from each individual were stained via a succinate dehydrogenase histochemistry procedure. Slides were allowed to

incubate in the SDH working solution at 37°C for 60 minutes. Slides were then washed in distilled water and mounted on an aqueous mount. Another subset of slides was incubated in an SDH working solution without phenazine methosulphate, as a negative control. Slides were photographed at a magnification of 100X under a microscope (Olympus BX4, Olympus Australia, Queensland), and red muscle fibers (dark purple/blue) were counted and their diameters measured.

4.3 COVID-19 Statement

Unfortunately, due to the COVID-19 pandemic, this chapter was removed from my PhD thesis. The tissue samples were collected prior to our lockdown at James Cook University (March 2020) and remained in our -80°C freezer. I was not able to begin sectioning the samples until September. When we started sectioning the samples, they all exhibited sufficient damage from ice crystal formation. Generally, sectioning of tissue should occur shortly (i.e., usually within a week) after tissue collection, as the activity of the enzymes significantly declines over time. Keeping samples for long durations at low temperatures also increases the risk of ice artefacts in the tissues, but it was a risk I had to take. My plan was to sample, section, and stain during each day. However, given the circumstances of the pandemic this was not possible (myself and JCU's histologist were not allowed access to the laboratories until August), and due to the time pressure to finish my thesis, I was not able to redo this chapter in time for submission.

Despite the setback, it is very important for me to keep the idea, proposed methodology, and relevance of this chapter in my thesis, as the morphological analysis of muscle tissue development acts as a clear transition from **chapter 3**, where I determined that growing larger is likely a key contributing factor for the high performance exhibited by tropical reef fishes. Therefore, it was of interest to better understand how tropical reef fishes develop their muscle tissues in comparison with other groups of fishes. If my stated hypotheses are correct, then 1) the anemonefish likely begins developing red muscle as an embryo; whereas, other fishes develop adult red fibers as late-stage larvae, and 2) the rate of red muscle tissue synthesis is likely much higher than has been determined in other groups of fishes. Given this predicted fast growth and development of highly metabolically active tissues, the larvae would have to energetically support such rapid ontogenetic changes. This transitions to **chapter 5**, where I investigate the energetic demands supporting swimming and growth across ontogeny for a coral reef fish species.

Chapter 5: The Exercise Physiology of Larval Reef Fishes Over Early Ontogeny

5.1 Summary

Swimming performance, growth and basic maintenance of tissues are a few critical metabolic functions relying on oxygen uptake. Marine teleost larvae are characterised by their high mass-specific oxygen uptake rates, which are significantly higher than juveniles and adult conspecifics. These high rates are indicative of the high metabolic costs to grow and develop muscle tissue and organ systems. Oxygen uptake rates therefore change as larvae develop, suggesting that how oxygen is transported and delivered (e.g., haemoglobin and other globins) throughout the body also changes. The current knowledge-base on marine teleost larvae has focused on temperate larvae during periods of unforced activity indicative of routine metabolic rates. Very little is known about oxygen uptake rates that support swimming. For tropical reef fishes that have high swimming performance critical for dispersal and finding a reef, the oxygen uptake rates that support such performance is widely unknown. The aim of this chapter was to use swimming respirometry techniques to measure oxygen uptake rates over the entire larval stage of a reef fish (*Amphiprion melanopus*). Additionally, I investigated changes in haemoglobin and other primary globins for oxygen transport/delivery (e.g., myoglobin, cytoglobin and neuroglobin) gene expression to determine whether any changes in oxygen uptake rates over larval development may have a genetic basis. I found that *A. melanopus* larvae have the highest oxygen uptake rates than any other teleost, likely attributed to the highly developed nature of these larvae at hatch, and need to support fast swimming. Haemoglobin, as well as the genes of other globins associated with oxygen delivery and storage (e.g., myoglobin, cytoglobin, and neuroglobin) were expressed the highest when swimming was at its greatest and right before the fish settled onto the reef. This may suggest that larval reef fishes change how oxygen is delivered and stored in their body in response to the hypoxic nature of coral reefs. Taken together, coral reef fish larvae require high oxygen levels to support high swimming performance so they can find and swim towards a reef, and alter the genetic expression of proteins that transports and delivers oxygen, in order to successfully and rapidly prepare for the hypoxic conditions of their new reef home.

Associated Publication

Downie AT, Lefevre S, Illing B, Harris J, Jarrold M, Cowman PF, McCormick MI, Nilsson G, Rummer JL (*in preparation*) The Exercise Physiology of Larval Reef Fishes Over Early Ontogeny. Science Advances (Target)

Data availability: Data present in this chapter are available from the Research Data Repository (Tropical Data Hub) at James Cook University. DOI: https://doi.org/10.25903/842e-ha35

5.2 Introduction

Patterns in Oxygen Uptake Rates during Larval Stages of Marine Fishes

Understanding an animal's metabolism, defined as "the biochemical processes that enable organisms to transform energy and materials from the environment into biological structures and functions (e.g., locomotion) to sustain life and perform activities" (Killen et al., 2010), is an integral aspect of physiology. An animal's metabolism is a complex series of biochemical pathways and associated organ systems, and thus, it is difficult for there to be a universal method that encompasses all of these systems. Oxygen (O_2) uptake rate ($\dot{M}O_2$) has been widely used as a proxy for metabolic rate given the integral roles with other performance and growth metrics such as tissue growth, protein synthesis, energy production, basic maintenance, locomotion, to name a few. Since the early 1900s, there has been much interest in using $\dot{M}O_2$ as a proxy for metabolic rate in fishes, especially during early life history stages (e.g., larval and juveniles) (Peck & Moyano, 2016). This is largely because ontogeny is a complex series of biochemical and physiological processes, and, while there is significant variation in how larval fishes develop among the thousands of extant species, the role of metabolism (e.g., oxygen demand) powering these processes is a common characteristic of development.

The overwhelming majority of research on respiratory physiology of larval fishes has focused on how $\dot{M}O_2$ scales with size (usually body mass), to better understand how metabolic rate changes as larvae grow and develop, and the energy required for ontogenetic milestones (e.g., development of specific organs such as gills and stomach) to be supported. In short, early studies have argued that metabolism and body mass universally scale among teleost fish larvae in specific ways, such as isometric (Giguère et al., 1988), curvilinear (Bochdansky & Leggett, 2001), and biphasic (Post & Lee, 1996). However, it is important to note that decades of research have shown that there is likely no universal trend or scaling exponent to communicate how metabolism and growth scale among *all* marine fishes, and this relationship is likely also highly species specific.

Generally, absolute $\dot{M}O_2$ (unit of O_2 [nmol, mg] individual larva^{-1} unit of time [hr, day]$^{-1}$) increases linearly (isometric or allometric with a scaling exponent ranging from 0.6 to 1) with size (dry or wet mass). This trend has been determined for larvae spanning a wide variety of fish species, examples including temperate pelagic fishes (e.g., Atlantic herring *Clupea harengus* Holliday et al., 1964; Kiorbøe et al., 1987; Atlantic tuna *Thunnus thynnus* Miyashita et al., 1999), flatfishes (e.g., Japanese flounder *Paralichthys olivaceus* Sumule et al., 2003; sole *Solea solea* Day et al., 1996; halibut *Hippoglossus hippoglossus* Finn et al., 1995), temperate demersal fishes (e.g., Atlantic cod *Gadus morhua* Finn et al., 2002; Peck & Buckley, 2008); haddock *Melanogrammus aeglefinus* Lankin et al., 2008), subtropical

coastal fishes (gilthead seabream *Sparus aurata* Parra & Yúfera, 2001), and tropical reef fishes (e.g., rabbitfish, *Siganus randalli*; Nelson & Wilkins, 1994). This linear increase in $\dot{M}O_2$ is due to increases in energetic demands associated with increased activity as fishes get larger and the need to consume more oxygen due to rapid tissue differentiation. A critical point here is that all of these studies investigated routine metabolic rate (RMR), which is estimated by measuring oxygen uptake rates of an unprovoked or relaxed individual (Peck & Moyano, 2016), generally on conscious larvae in groups of 3-20 individuals; although, some studies have measured $\dot{M}O_2$ on anaesthetized larvae (e.g., Kiørbe et al., 1987). It is also important to note that RMR does not include any forced activity associated with swimming, which is predicted to represent much of a larval fish's energy budget (described below). Given the numerous anatomical and physiological changes larvae undertake during their early life history that occur regardless of size, a more accurate representation of metabolism over larval fish development would be to compare $\dot{M}O_2$ with age.

Correlating mass-specific $\dot{M}O_2$ (e.g., mg O_2 kg^{-1} hr^{-1}) with age more clearly shows how changes in energy demands are related to specific ontogenetic milestones (e.g., opening of mouth, notochord flexion, etc.). Several studies found that RMR changes at specific stages of development. As fishes grow, different tissues contribute to different amounts of $\dot{M}O_2$ over ontogeny. For example, in tuna (*T. thynnus*), there are distinct patterns in RMR when yolk sac larvae switch to endogenous feeding, upon notochord flexion, and then upon the onset of metamorphosis into juvenile stages (Myashita et al., 1999). Similar distinct changes in RMR across early ontogeny have also been observed in rabbitfish (*Siganus randalli;* Nelson & Wilkins, 1994), red seabream (*Pagrus major*; Oikawa et al., 1991), and ocellate puffer (*Leiodon cutcutia*; Miyashima et al., 2012). However, there are exceptions such as gilthead seabream (*S. aurata*), which exhibits a linear increase in RMR with age; gilthead seabream get most of their energy from endogenous lipid stores throughout their larval stage (Rønnestad et al., 1994). Generally, for larvae that hatch from pelagic eggs, most energy is derived from endogenous yolk-sac reserves, and upon absorption of this reserve, larvae switch to exogenous feeding at which point RMR generally increases with the increased activity required to find prey (e.g., Davenport & Lönning, 1980; Davenport, 1983; Evans et al., 2006). The time when the caudal fin forms (notochord flexion) coincides with gill formation and large axial muscle synthesis, which results in marked increases in influential swimming (**chapter 2**; Downie et al., 2020) and RMR (e.g., cod *G. morhua* Hunt Von Herbing & Boutilier, 1996; seabream *Pagrus major* Ishibashi et al., 2005; rose snapper *Lutjanus guttatus* Parra et al., 2016). However, RMR decreased in dusky kob (*Argyrosomus japonicas*) due to the energetic demands around that milestone of notochord flexion (Edworthy et al., 2018). Upon transitioning to juvenile stages, mass-specific $\dot{M}O_2$ generally decreases in response to several physiological changes such as the transition from cutaneous to branchial respiration, increased growth and protein synthesis, decreased surface area to volume ratios, and the

decrease in size of highly metabolically active tissues relative to the rest of the body (Holliday et al., 1964; Oikawa et al., 1991; Day et al., 1996, Killen et al., 2007). Additionally, behavioural changes (e.g., transition from pelagic larvae to benthic or demersal juveniles) in swimming also contribute to differences in how $\dot{M}O_2$ changes over ontogeny (e.g., de Silva & Tytler, 1973). High inter-individual variability of how $\dot{M}O_2$ scales with mass is likely due to lacking standardized methods for determining $\dot{M}O_2$, which limits understanding of mass-dependent metabolic rates of larval fishes (Killen et al., 2007). Additionally, the vast majority (>95%) of respirometery studies on larval fishes to date have involved temperate species, while we have very little knowledge as to how $\dot{M}O_2$ changes with size and ontogeny for subtropical and tropical species. Taken together, it is important to place emphasis on the importance of measuring $\dot{M}O_2$ across the larval phase at distinct ontogenetic stages and furthermore under various stages of activity.

Challenges of swimming respirometry of marine teleost larvae

Very few studies have investigated cost of swimming during activity for marine teleost fish larvae. Most of the current knowledge-base on active metabolic rates have used freshwater fishes, such as cyprinids (e.g., Dabrowski, 1986; Kaufmann, 1990; Kaufmann & Wieser, 1992; Wolfgang Wieser & Kaufmann, 1998). As mentioned previously, what is currently known regarding active $\dot{M}O_2$ of the earliest life stages of marine teleost fish larvae is based on RMR measurements that do not take into account swimming, which can require up to 70% of a fish's energy budget (Herbing & Boutiler, 1996). Historically, the challenges of measuring respiration rates of larval marine fishes during activity are mainly due to imprecise equipment (e.g., older electrodes cannot detect oxygen uptake of an individual larva during exercise), small sizes of temperate larvae (e.g., cod, flatfishes and herring, which make up most of the current body of literature), and swim respirometers that cannot be scaled down to small enough sizes (Steffensen, 1989; Peck & Moyano, 2016). Few studies have accounted for spontaneous activity in static respirometry chambers (e.g., Lasker & Theilacker, 1962), and there have been attempts to justify maximum oxygen uptake measurements during RMR tests as the highest 5-10% of $\dot{M}O_2$ measurements (e.g., Edworthy et al., 2018; Geist et al., 2013); however, these estimates would significantly underestimate $\dot{M}O_2$ during activity, as higher rates of prolonged forced activity would likely incur a higher metabolic cost than spontaneous activity within a respirometry chamber. To my knowledge, the only study to investigate active metabolic rates in fish larvae (sculpin *Myoxocephalus scorpius*, ocean pout *Macrozoarces americanus*, and lumpfish *Cyclopterus lumpus*) over ontogeny is by Killen et al., (2007). Using a combination of chase methods and constant velocity tests, maximum oxygen uptake rates were measured after the fish fatigued (i.e., during excess post-exercise oxygen consumption; EPOC) and could be used to determine absolute aerobic scope (whole energy budgets for all aerobically

driven tasks; Killen et al., 2007). Based on this work, it was determined that larval fishes have a low scope for activity, leaving little room for homeostatic maintenance in response to environmental change. This work may be representative for temperate fishes that are generally poor swimmers throughout most of their larval stage, but it may not represent tropical reef fishes that have a high capacity for swimming performance throughout ontogeny.

Oxygen uptake rates of tropical reef fishes.

Despite the expansive literature on swimming performance of tropical reef fish larvae, particularly settlement stage fishes, and the importance of swimming for dispersal processes, connectivity, and recruitment among reef fish populations, we know very little about the energetic demands that support swimming performance. Similar to temperate fishes, the current knowledge base on tropical reef fish energetics is based on RMR measurements, with most on settled juvenile fishes (e.g., McLeod et al., 2013; Killen et al., 2014; Hess et al., 2017), with little information available on how $\dot{M}O_2$ scales over ontogeny (see Nelson & Wilkins, 1994). However, in a landmark study, Nilsson et al., (2007a) used swimming respirometry methods to determine that pre-settlement reef-associated damselfishes (*Pomacentrus amboinensis* and *Chromis atripectoralis*) had very high active metabolic rates and low tolerances to hypoxia, and upon settling onto a reef, became very tolerant to hypoxia and exhibited a decrease in $\dot{M}O_2$. Adult and juvenile reef fishes experience nocturnal hypoxia events, which are associated with coral and benthic respiration, and as a result are hypoxia tolerant (Nilsson et al., 2007b). Therefore, there appears to be a trade-off between high $\dot{M}O_2$ during activity and low $\dot{M}O_2$ to be hypoxia tolerant, which is hypothesized to be related to opposing demands of oxygen-carrying properties of haemoglobins (described below; Nilsson et al., 2007a,b). Based on the available literature (e.g., Fisher et al., 2005; Nilsson et al., 2007a,b) and findings from **chapter 3 (**Downie et al., 2021a**)**, the ability to find a suitable reef relies on high swimming capabilities, which is likely constantly supported energetically and mainly aerobically. However, upon finding a reef, the energy demands and how energy is transported throughout the body likely shift to tolerate nocturnal hypoxia events. This may be due to switching in haemoglobin isoforms during the pelagic phase in preparation for a transition to reef life.

The multiple haemoglobins of fishes

Haemoglobin (Hb) is a protein contained within red blood cells (erythrocyte) that consists of polypeptide chains (globins) that contain a prosthetic group (heme) which allows the binding of oxygen (de Souza & Bonilla-Rodriguez, 2007). The main function of haemoglobin is to transport oxygen from the site of gas exchange (i.e., the skin, lungs, or gills, depending on the species and life stage) to peripheral tissues (e.g., muscles and organs; de Souza & Bonilla-Rodriguez, 2007) and vice-versa for

metabolic waste products (e.g., carbon dioxide). Unlike in mammals that have one type of haemoglobin expressed at any given ontogenetic stage, fishes, reptiles and amphibians express numerous isoforms of haemoglobins simultaneously (known as haemoglobin polymorphism). An isoform is defined as a protein from the same family with similar or distinct functions. The adaptive significance of haemoglobin polymorphism is that, across ontogeny, oxygen demands change (e.g., depending on environmental conditions) and therefore, distinct isoforms of haemoglobins are apparent at certain developmental stages in response to selective pressures under unstable conditions (de Souza & Bonilla-Rodriguez, 2007). This relates to the binding capacity different haemoglobin isoforms have for oxygen.

In general, optimal Hb-O_2 affinity is dictated by a trade-off between oxygen loading at respiratory surfaces and oxygen unloading at tissues, and the degree by which haemoglobin can bind oxygen can depend on the ratio between cathodic and anodic haemoglobin isoforms (Storz et al., 2019). For example, across ontogeny, some species show dramatic shifts in proportions of cathodic and anodic haemoglobins in response to environmental changes. Cathodic haemoglobin isoforms have a high affinity for oxygen, and cathodic isoforms generally lack a Bohr effect, where decreases in pH decrease Hb-O_2 affinity, which is beneficial for unloading oxygen at the tissue level (Storz et al., 2019). Having a high ratio of cathodic isoforms can safeguard a species under hypoxic conditions, as high Hb-O_2 affinity facilitates oxygen uptake at the respiratory surfaces despite an oxygen-limiting environment (Imsland et al., 1997). In contrast, anodic haemoglobin isoforms have a low affinity for oxygen and a pronounced Bohr effect (Storz et al., 2019). Therefore, under normoxic conditions, a high ratio of anodic isoforms facilitate efficient oxygen delivery to tissues, thus presumably supplying adequate energy for growth and swimming. Fishes express various haemoglobin isoforms with varying degrees of anodic and cathodic components, broadening the spectrum of environmental conditions (e.g., temperature, oxygen, salinity, pH, etc.) in which they can live. However, a common pattern among fishes is that active, usually pelagic, species have lower haemoglobin oxygen affinities than less active benthic species, which may enable active species to enhance oxygen delivery to actively metabolising tissues (e.g., muscles; Koch, 1982). Indeed, the vast majority of research on haemoglobin isoforms in fishes has been on highly active groups, mainly salmonids (Giles & Vanstone, 1976).

Much of the research on fish haemoglobins has been on salmonids, as they can have up to 18 electrophoretically distinct haemoglobin isoforms during their life cycle (Giles & Randall, 1980). In Coho salmon (*Oncorhynchus kisutch*) at least 3 out of the possible 18 haemoglobin isoforms are always present, and the rest are present during distinct ontogenetic stages: embryonic, freshwater fry and smolt, and adult (Giles & Randall, 1980). It is important to note that certain haemoglobin isoforms that are specific to ontogenetic stage have varying degrees of oxygen-binding affinity. For example, Coho salmon fry

possess haemoglobins with high oxygen binding affinity (i.e., cathodic), likely in response to living in habitats with low oxygen levels (e.g., riverbed sediments; Giles & Vanstone, 1976; Giles & Randall, 1980). During smoltification (i.e., physiological acclimatization to seawater entry), adult haemoglobin isoforms begin to increase (i.e., shifting toward more anodic haemoglobin isoforms). It is understood that this occurs to ensure efficient oxygen release to the tissues during rapid changes in environmental conditions (e.g., temperature, salinity) and to compensate for the metabolic acidosis incurred during bursts of high-intensity swimming and during spawning migrations (Vanstone et al., 1964; Giles & Randall, 1980; Sullivan et al., 1985). A similar ontogenetic shift in haemoglobin isoforms has also been extensively studied in Chinook salmon (*Oncorhynchus tshawytscha*) (Koch, 1982; Basaglia, 2004). It should be noted that the onset or regulation of ontogenetic haemoglobin isoform shifts are not influenced by environmental stimuli (e.g., temperature, salinity, O_2, pH, etc.), but instead they are 'genetically hardwired' into the species' developmental life history and likely mediated by thyroid hormones (Giles & Randall, 1980). While much of the research focusing on haemoglobin isoforms in fishes are on salmonids, other fish groups exhibit a similar isoform switching pattern when experiencing environmental hypoxia.

Interspecific differences in haemoglobin isoforms, hypoxia tolerance, and ability to switch between isoforms is largely correlated with the degree by which a species' environment experiences diurnal changes in hypoxia. For example, flounders that live in coastal habitats have higher oxygen affinity haemoglobins due to chronic, daily hypoxic conditions they experience than closely related deep sea plaice (Weber & de Wilde, 1976). Triplefin species that live in tide pools that experience variable oxygen levels have a greater number of high affinity cathodic haemoglobin isoforms when compared to fishes living in deeper, stable habitats species, which tend to possess more anodic haemoglobin isoforms (Brix et al., 1999). A similar pattern has been observed in sculpins, as hypoxia tolerant species have lower RMR than hypoxia intolerant species and a higher ratio of cathodic to anodic haemoglobin isoforms (Mandic et al., 2009). Lastly, some species of cichlids and red drum (*Sciaenops ocellatus*) are capable of upregulating higher affinity haemoglobin isoforms during hypoxia acclimation (Rutjes et al., 2007; Pan et al., 2017). However, there are some examples where haemoglobin isoform switching does not occur in response to environmental change, such as the blue-spotted mudskipper (*Boleophthalmus pectinirostris*, Storz et al., 2020) and mangrove rivulus (*Kryptolebias marmmoratus*, Tunnah et al., 2021). Taken together, and given the wide evidence for haemoglobin isoform switching and ontogenetic shifts in haemoglobin isoforms, it is highly possible that tropical reef fish larvae switch haemoglobin isoforms, from low affinity during their pelagic phase to high affinity upon settlement to be better suited for a reef habitat that experiences daily, nocturnal hypoxia events.

Aims

The broad goal of this chapter was to investigate the mechanisms underpinning oxygen uptake and delivery that support swimming over the entire larval duration of the anemonefish (*Amphiprion melanopus*). This species is an ideal candidate to explore metabolic ontogeny of coral reef fish larvae, as they are among the few reef fish species in which we have been able to close the life cycle under a laboratory setting. Since most reef fishes have a similar life history pattern (i.e., demersal, site-attached adults and a pelagic larval stage), the patterns in how oxygen uptake rates change during ontogeny and the magnitude of oxygen uptake rates over larval development may be representative of other reef fishes. While these fishes possess well-developed muscle (predicted from **chapter 4**) and organ systems and have a high aptitude for complex behaviours, such as swimming immediately upon hatch, anemonefish must also be able to physiologically support this performance and maintenance of complex, metabolically-active tissues. There were two specific aims. Firstly, to investigate daily measurements of active metabolic rates and swimming speeds over the entire larval period of the anemonefish, I use swimming respirometry techniques to measure oxygen uptake rates during periods of forced activity. Active metabolic rates allow me to better predict overall energy budgets (e.g., aerobic scope) for a species, and to date, this is unknown for any tropical coral reef fish species during early ontogeny. Secondly, using a molecular approach, I will explore changes in gene expression patterns related to Hb (e.g., isoform switching) and other oxygen dependent pathways across ontogeny. I am also interested in investigating the expression patterns of genes that code for other proteins that are critical for oxygen transport and storage, such as neuroglobin (stores oxygen in the brain), cytoglobin (oxygen storage in connective tissues), and myoglobin (transports oxygen into muscle tissues). Active pelagic larvae must make a rapid transition to a benthic lifestyle as a juvenile, and I predicted they must also make rapid changes to expression patterns of genes associated with oxygen transport as consequence. Following hypothesis 3 of this thesis, I predicted: i) that the anemonefish in my study support high swimming capabilities and high tissue growth during early ontogeny with high oxygen uptake rates, and ii) high oxygen uptake rates are due to haemoglobin isoforms with low oxygen affinity, thus supporting high oxygen unloading at tissues, and then, upon metamorphosis, a switch in isoforms characteristic of high oxygen affinity prepares anemonefish for hypoxic conditions that are common on the reef at night. Additionally, I predict a similar pattern in the expression patterns for genes related to other globins (e.g., myoglobin, cytoglobin, and neuroglobin) if I find detectible changes from the RNA extractions. Additionally, I predicted that genes associated with oxygen delivery, such as haemoglobin, increase in regulation as the larvae approach the point of settlement (i.e., near the end of their larval duration) in response for preparation for the transition to the hypoxic conditions of the reef, requiring enhanced delivery of oxygen to tissues. From my results, I will be able to evaluate daily changes in oxygen uptake

rates that support athletic swimming capabilities, growth and development for a candidate coral reef fish species as well as determine the physiological mechanisms underpinning their transition to a hypoxic reef environment. This transition is a critical aspect of reef fish life history, and studying the physiological and genetic processes underlying this event will advance our understanding of how larval reef fishes successfully recruit to adult populations.

5.3 Materials and Methods

Note: The following ethics statement and protocol for animal husbandry and larval rearing is repeated, verbatim, from **Chapter 4.**

All housing of adult anemonefish rearing of larvae, and experimentation occurred at James Cook University campus at the Marine and Aquaculture Research Facilities Unit (MARFU). All husbandry and experimentation methods were approved by James Cook University's animal ethics committee (ethics number A2425).

5.3.1 Husbandry of study species

Adult breeding pairs of the cinnamon anemonefish (*Amphiprion melanopus*) have been established at MARFU since 2015 but were originally captured on the Great Barrier Reef by commercial divers (Cairns Marine). For this study, adult pairs were maintained in 60L flow-through outdoor aquaria at MARFU. Natural summertime water conditions were maintained throughout the duration of the experiment (temperature = 28°C, salinity = 33ppt), and fishes experienced natural photoperiods. Adults were fed twice daily using pellet food (NRD G12 Inve Aquaculture Inc., Salt Lake City, USA). Within each tank there was half a terra-cotta pot for shelter and a place for adults to lay their eggs. Tanks were cleaned weekly to maintain good water quality (ammonium levels kept below 0.04ppm, and nitrate levels were kept within 10-40ppm).

Adults generally lay eggs every two weeks, and eggs generally hatch 7-8 days later. On the day prior to predicted hatching, the terra-cotta pot was removed from the adult tank and promptly replaced with a blank pot and then transported in water to a 100L flow-through (water parameters: temperature = 28°C, salinity = 33ppt) larval rearing tank in a separate indoor room. A summer photoperiod 13h light to 11h dark was maintained in the larval rearing room during the experimentation period. An air stone was placed under the eggs to simulate parents aerating the eggs, and promote hatching. Upon day at hatching to 5 days post hatch (0-5dph), larvae were fed rotifers (*Brachionus* sp.) at a concentration of 20 individuals ml^{-1}. Additionally, from hatch to 3 dph, 3ml of algal paste (*Nannochloropsis* sp.) was added to the tanks to feed rotifers and provide shelter to the larvae from light-stress. From 3dph-9dph, larvae were

fed freshly hatched *Artemia* sp. naupli *ad libtum*. During feeding, water was switched off for 1 hour to prevent food and algae from being removed from the tank, allowing larvae to have adequate time to feed. Water was switched back on to flush the system and maintain water quality.

5.3.2 The swimming respirometer

Each fish was swum using a custom-built, glass, Blazka-style swimming respirometer (volume (V)=125ml; length (L) = 14.5cm; diameter (⌀) = 2.7cm), which permits a simultaneous measure of oxygen uptake rates ($\dot{M}O_2$) while an individual swims at any given speed (Figure 5.1). The swimming respirometer was calibrated prior to experimentation using a high-speed camera (Casio Exilim High Speed Camera) and passive particles. To reduce the volume of the respirometer, providing a more accurate $\dot{M}O_2$ measurement for the size of animals swum, an insert (⌀ = 2.6cm, L= 8.5 cm) was placed in the respirometer to create the working section (Figure 5.1). The working section of the respirometer (V = 38 ml, L= 4.5cm, ⌀ = 2.7cm) fit a smaller chamber, where the individual fish swum (Figure 5.1). This smaller chamber (V = 3.5ml, L = 2cm, ⌀ = 1.5cm) was fitted with a flow straightener (made from capillary tubes of ⌀ = 1.1mm, L = 40mm) to mitigate micro-turbulent flow, and a downstream mesh barrier to prevent the individual fish from being sucked into the propeller (mesh ⌀ = 0.415 mm) (Figure 5.1). The swimming chamber was large enough for an individual fish to swim in any direction comfortably (i.e., to mitigate stress of enclosure) and prevented blocking effects (<5%) which would alter the flow within the chamber. An external flush pump was used to deliver clean seawater (temperature, T = 28°C; pressure, P = ~1 bar; salinity, S = 33ppt, dissolved oxygen, DO = ~6.1 mg ml^{-1}) to the system in between measurement periods (see *Experimental protocol* for details). Oxygen uptake rates (mg O_2 ml^{-1}) and T (°C) were simultaneously measured using respective probes (oxygen probe: OXROB3 Robust Oxygen Probe, PyroScience, Achen Germany; T sensor: TSUB36 Shielded submersible temperature sensor, Pyroscience, Achen, Germany) (Figure 5.1). Oxygen probes were calibrated to 100% air saturation using fully aerated seawater (T = 28°C, S = 33ppt, P = 1bar, DO = 6.1mg O_2 ml^{-1}) and to 0% oxygen saturation (T = 28°C, S = 33ppt, P = 1 bar, DO = 0mg O_2 ml^{-1}) using sodium sulphite (Na_2SO_3; UNIVAR Analytical Reagent, Ajax Finechem, New South Wales, Australia). Oxygen and temperature probes were connected to a Firesting oxygen meter (4-channel optical oxygen meter, Pyroscience, Achen Germany), which constantly measured both of these variables throughout each experiment (1 Hz, or s^{-1}). Water temperature was maintained at experimental conditions (T = 28°C), even at high water velocities (Figure 5.1), by means of a temperature jacket (V = 85ml, L = 6.5 cm). Prior to all swimming trials, all components of the swimming respirometer were washed in a 10% bleach solution to kill any bacteria in the system.

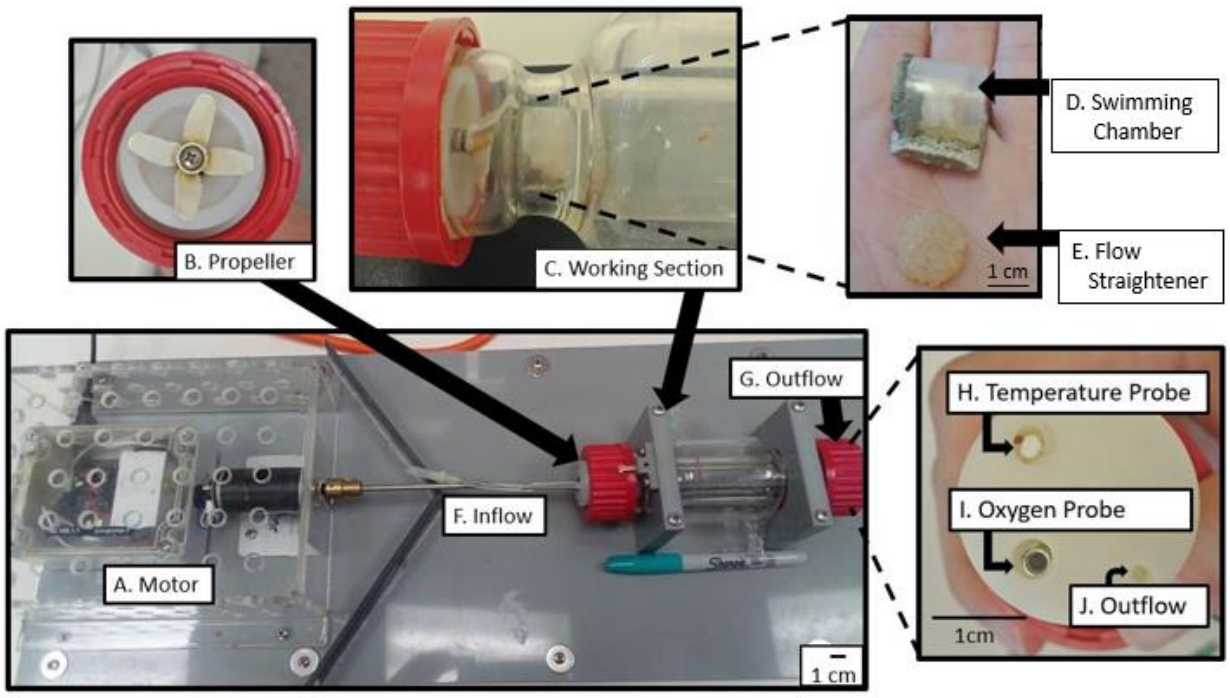

Figure 5.1 The swimming respirometer used to swim larval cinnamon anemonefish (*Amphiprion melanopus*) from hatch until settlement. A motor (A) rotates an axle at variable speeds (1-40 cm s^{-1}) via a dial on a control box, which creates a current in the respirometer by a propeller (B). The working section (C) is created by placing an insert into the swimming respirometer to reduce. The working section houses the swimming chamber (D) where the individual fish swims. A flow straightener (E) mitigates micro-turbulent flow, and mesh screen prevents the individual fish from being sucked into the propeller. Water is replenished into the respirometer via an inflow pipe (F) and flushes out of the system via an outflow pipe (G). Each insert is outfitted with a temperature probe (H), oxygen probe (I), and an outflow (J). The marker (approximately 14cm in length) was added for scale.

5.3.3 Experimental protocol

The experiment was designed to measure ontogenetic changes in oxygen uptake rates ($\dot{M}O_2$) during periods of activity for the anemonefish. Since I am measuring $\dot{M}O_2$ on active larvae, I can easily get estimates of maximum metabolic rate (MMR; highest oxygen uptake rates resulting from the onset of fatigue), standard metabolic rate (SMR; basic maintenance costs) and aerobic scope (AS; total energy budget for all aerobically driven tasks) from each swimming experiment. Calculating these metrics will be provided in more detail below, but it is important to note here that each developmental age undergo the same protocol. These experiments were conducted daily on larvae from time at hatch (0dph) until time at settlement (9dph) (n=8-10 larvae per developmental age). Since this study relies heavily on ontogenetic changes in metabolic rate, for consistency, all larvae used in this experiment came from the same parent anemonefish breeding pairs to mitigate parental effects, all larvae were reared in the same larval rearing aquaria to eliminate any potential tank effects, and all larvae were individually swum in the same swimming respirometer. Multiple clutches were used to achieve the sample size for each developmental age, but keeping the breeding pairs consistent mitigates any potential differences in physiology between offspring of different breeding pairs.

All experiments were performed in a dark experimental room at MARFU separate from the larval rearing room to prevent external stimuli from influencing the experiment; however, a red headlamp (600 lumens, Ledlenser MH10; Ledlenser Australia, New South Wales) was used so I could see during the experiments. Prior to each individual experiment, background respiration (i.e., oxygen uptake rates of bacteria) was measured for ten minutes. To mitigate bacterial respiration, the seawater (28°C) used for all experiments was filtered using an ultra-violet filter (Blagdon Pro 24W ultra-violet clarifier, Dreative Pumps, South Australia, Australia). Larvae were fasted for at least 12-15 h before experimentation to ensure they were in a post-absorptive state and there would be no influence of digestion (i.e., specific dynamic action) on oxygen uptake rates (Killen et al., 2007). Then, an individual larva was gently removed from the rearing tank, placed in a black covered bucket, and gently transported to the experimental room (<2 min). The individual larva was then placed into the swimming chamber by gently pouring it in and then quickly positing it into the working section of the swimming respirometer (Figure 5.1). This was performed in a separate tank (‘preparation tank’) so the entire swimming respirometer could be sealed underwater to prevent air bubbles from building up in the system. The swimming chamber was then gently removed from the ‘preparation tank’, and then the motor was attached (Figure 5.1). Each individual fish was allowed to habituate to the chamber and rest from handling/transport stress for 60 min, under constant, gentle flow conditions at a water velocity equivalent to 1 body length per

second (BL sec^{-1}; a subsample of individuals was measured per species [total length; snout to tip of caudal fin] to provide an overall estimate for body length).

Individual larvae underwent a stepped velocity test, post-habituation, to measure critical swimming speed (U_{crit}), a test designed to estimate the aerobic capacity of fishes. Every 20 min, the water velocity in the chamber was increased by 1 BL sec^{-1} until the individual fish fatigued, as indicated by impingement on the downstream barrier. Critical swimming speed was calculated using the following formula:

$$U_{crit} \text{ (cm sec}^{-1}) = Vf + (T/t)*Vi$$

Where, vf is the penultimate speed (cm sec^{-1}), T is the time swum at the fatigue speed, t is the time interval (20 minutes) and Vi is the velocity increment (approximately 1 BL sec^{-1}) (Brett, 1964). At each swimming speed, $\dot{M}O_2$ was measured using intermittent flow respirometry, consisting of a 20-minute measurement period (i.e., the time interval portion of the U_{crit} protocol), followed by a 3-minute flush period (i.e., to replenish the swimming chamber with clean, fully-aerated seawater, which lasts until the water velocity increases to the next speed). The flush period was long enough for oxygen saturation within the swimming chamber to be replenished to 100% air saturation (DO=6.1mg O_2 ml^{-1}). However, oxygen within the swimming chamber was never allowed to fall below 90% air saturation to prevent oxygen uptake rates of the fish from being influenced by hypoxia. Upon completion of their respective U_{crit} protocol, the fish was removed from the respirometer, euthanized in an ice bath, and a subsequent reading of respiration by any accumulated bacteria in the chamber introduced by the fish was performed for 10 minutes.

Text files from the Firesting were imported and analysed in LabChart (ver 8, AD instruments, New South Wales, Australia) to calculate $\dot{M}O_2$ at each swimming speed. The oxygen uptake rate ($\dot{M}O_2$) at each 20-minute interval was calculated as:

$$\dot{M}O_2 \text{ (mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}) = S \bullet V_{resp} M^{-1}$$

where S is slope of the linear regression during the measurement period (mg O_2 s^{-1}), V_{resp} is the volume of the respirometer (minus the fish), and M is the mass of the individual fish (kg) (Rummer et al., 2016b). Background respiration was subtracted from each value of $\dot{M}O_2$. For each individual swimming experiment (i.e., for each individual larva per day), each value of $\dot{M}O_2$ was plotted against each swimming speed (cm sec^{-1}). The appropriate linear regression (1) or power curve (2) was fit through this relationship (von Herbing & Boutiller, 1996):

$$(1)\ R(u) = a + bu$$

$$(2)\ R(u) = a + cu^{b}$$

Where R(u) is an estimate of mass-specific oxygen uptake (mg O_2 $kg^{-1}h^{-1}$) at any given speed (u; cm sec^{-1}), a is the y-intercept, b is the slope of the equation (linear regression; equation 1) or scaling exponent (power curve; equation 2), and c is an estimated parameter (von Herbing & Boutilier, 1996). The y-intercept of this relationship between oxygen uptake rates and swimming speed provides an estimate of the fish's standard metabolic rate (SMR; mg O_2 kg^{-1} h^{-1}), which is an estimate of the basic metabolic functions of the animal at rest (u=0 cm sec^{-1}) (von Herbing & Boutilier, 1996). The maximum $\dot{M}O_2$ value when the individual fish fatigued, is an estimate of maximum metabolic rate (MMR; mg O_2 $kg^{-1}h^{-1}$), which is also an estimate of the maximum sustainable (i.e., aerobic) swimming speed. Aerobic Scope (AS; mg O_2 kg^{-1} h^{-1}) was calculated by subtracting SMR from MMR, and is the total amount of oxygen available to the fish to perform aerobically driven tasks beyond basic maintenance (e.g., swimming, finding food, avoiding predators, etc.). Aerobic scope was presented as both absolute AS (MMR-SMR) and factorial aerobic scope (FAS; SMR/MMR) for each developmental age. FAS shows the fold increase between rest and maximum $\dot{M}O_2$ (Killen et al., 2007).

5.3.4 RNA Extraction and Sequencing

A subset of 60 larvae from 4, 6, and 9dph groups were euthanized and preserved in individual 1.5ml vials containing RNAlater (Sigma, Germany) at -80°C until RNA extractions were performed. These developmental days were selected based on ontogenetic changes in oxygen uptake rates during those days that is predicted be caused by changes in haemoglobin-binding affinity (see results and Figure 5.3). Such a large sample of larvae were collected initially at each age because it was unknown how many larvae would need to be pooled together per replicate to extract enough RNA. I performed a trial run at the smallest sized larvae (4dph) using the RNA extraction procedure (described below) using different numbers of pooled larvae per replicate (1, 2, and 3 larvae pooled per replicate). I determined that one larva per replicate was sufficient to extract enough RNA, and for each developmental age, RNA was extracted from 6 larvae (n=18 total).

Using sterilised forceps, the RNAlater (Sigma Lifesciences, Germany) preserved larvae were removed from their individual vials, dabbed on aluminium foil to remove excess RNAlater and subsequently added to a powerbead tube (ceramic 2.8mm beads; Qiagen, Germany). A 600µl aliquot of a mix containing DTT/RLT (mix contains: 20µl 2M Dithiothreitol [DTT; Sigma, Germany] and 1 ml of RLT buffer [lysis buffer; Quigen, Germany]) was then added into each PowerBead tube. The samples were homogenized in a bead beater (Benchmark Scientific Beadbug D1030 Microtube Homogenizer;

Benchmark Scientific, USA) in two 45-second cycles at 5.5-6 m s^{-1} with 30 seconds in between cycles. The samples were then centrifuged (Mikro 185 centrifuge, Labgear, Australia) at 18 000g for 3 minutes. The resulting supernatant (hereafter, lysate) from each tube was then decanted into individual sterile, RNAse free 1.5ml tubes. Approximately 550 µl of 70% ethanol was added to each of the tubes containing lysate and mixed via pipette. From each tube, 700µl of lysate was loaded onto an individual RNeasy column (RNeasy Mini kit, Qiagen, Germany) and centrifuged at 8000 g for 15 seconds. The flow through was discarded. Then, the remaining sample from each tube was loaded onto their respective RNeasy column and centrifuged at 8000g for 15 seconds, subsequently discarding the flow through. To each RNeasy column, 350µl of RW1 (wash buffer; RNeasy min kit; Qiagen, Germany) was added and centrifuged at 8000g for 15 seconds, subsequently discarding the flow through. Then, 10µl of DNase stock – which was prepared beforehand by dissolving lyophilised DNase (1500kunits) in 550µl of RNase free water that had been divided into single use aliquots and stored at -20°C until use – was added to 70µl or RDD buffer (RNeasy mini kit; Qiagen, Germany) and mixed gently by pipetting. Exactly 80µl of DNase/RDD buffer mix was added to each RNeasy column filter and allowed to incubate at room temperature for 15 minutes. Then, 350µl of RW1 was added to each RNeasy column and centrifuged for 15 seconds, subsequently discarding the flow through. Then, 500 µl of RPE buffer (wash buffer; RNeasy mini kit; Qaigen, Germany) was added to each column and centrifuged at 8000g for 15 seconds, subsequently discarding the flow through. Another 500µl of RPE buffer was added to each column and centrifuged at 8000g for 2 minutes. Afterwards, each RNeasy column wsa placed on a new collection tube and centrifuged at 18000g for 1 minute. Each RNesy column was placed onto a clean labelled 1.5ml tube, 50µl of RNase free water was directly added to each column and centrifuged at 8000g for 1 minute. This step was repeated to acquire 100µl elutes. Elutes were stored at -80°C until shipped for sequencing. Samples were shipped on dry ice. Sequencing was performed by the Australian Genome Research Facility (AGRF) in Melbourne. From the total RNA provided, poly(A) tail selected mRNA libraries were prepared using the Illumina Stranded TruSeq kit. The 18 samples were sequenced on one lane NovaSeq6000 SP with 300 cycles (150bp PE), yielding 510,893,644 paired reads in total (per sample mean±S.D. = 28,382,980±5,130,537).

5.3.5 Bioinformatics

Analysis of the sequencing data were performed on the high-performance computing cluster Saga resources provided by UNINETT Sigma2. The raw sequence reads were trimmed using Trimgalore (0.6.2) with default parameters except for minimum length which was set to 40bp and quality score which was set to 20. The trimmed reads were quality checked using Fastqc (0.11.8) (Andrews, 2010) and based

on visual inspection of the resulting plots. The trimmed reads were then aligned to the genome of the closely related orange clownfish (*A. percula*) using STAR (2.7.6a; STAR genetics) in two-pass mode for discovery of novel splice junctions. The genome index was first prepared using genome and annotation file downloaded from Ensembl (release 102) with default parameters and --sjdbOverhang 149. Several trial runs were performed using different parameters to identify the command yielding an alignment rate, taking into consideration that the genome was from a closely related species and that reads were long. The final parameters used were:

--runThreadN 10
--twopassMode Basic
--peOverlapNbasesMin 12
--alignSJoverhangMin 10
--alignSJDBoverhangMin 10
--alignMatesGapMax 100000
--alignIntronMax 100000
--alignSJstitchMismatchNmax 1 -1 1 1
--alignSplicedMateMapLmin 30
--alignInsertionFlush Right
--chimSegmentMin 12
--chimJunctionOverhangMin 8
--chimOutJunctionFormat 1
--chimMultimapScoreRange 3
--chimScoreJunctionNonGTAG -4
--chimMultimapNmax 20
--chimNonchimScoreDropMin 10
--outSAMattrRGline ID:GRPundef
--quantMode GeneCounts
--outSAMtype BAM SortedByCoordinate
--limitBAMsortRAM 3000000000
--outBAMsortingThreadN 5
--outSAMattributes All

The average alignment rate across samples of uniquely mapped reads was 77.5±1.3%. Counting for downstream expression analysis was performed using the featureCounts tool from Subread (2.0.1) treating alignments as paired and reverse-stranded (-p -s 2), not counting chimeric alignments (-C), counting at the feature level exon (-t exon) but summarising at the meta-feature level gene (-g gene_id). Multipmapping and multi-overlapping reads were discarded, but singletons were allowed. Of the alignments output by STAR, the average assignment rate across samples was 72.1±0.9%.

Analysis of differential gene expression was performed on the gene count matrix from featureCounts using the DEseq2 package (Love et al., 2014; ver. 1.28.1) with R (ver 3.6.1. http://www.R-

project.org/). For plotting of gene expression, data were normalised using the trimmed mean of M-values method (TMM) in the EdgeR package (Robinson et al., 2010; ver. 3.30.3).

5.3.6 Statistical Analyses

All statistical analyses were performed in R (ver 3.6.1. http://www.R-project.org/). All data were checked for normality using diagnostic plots. Data were analyzed using a one-way analysis of variance, with response variable being either U_{crit}, SMR, MMR or AS, and age was a categorical variable. Differences between ages were determined with a Tukey's post hoc test (α=0.05).

5.4 Results

As cinnamon anemonefish develop, post-hatch, their swimming performance increases significantly and in distinct phases with age ($F_{9,91}$ = 61.05, p<0.001; Figure 5.2, Table 5.1, 5.2). Upon hatch (0dph), larvae are capable of critical swimming speeds (U_{crit}) of 1.04±0.02 cm s^{-1} (mean ± standard deviation or error). Swimming performance then increases by 44% between hatch and the first phase at 1-3 dph (5±0.03 cm s^{-1}; Figure 5.2). The next increase in swimming performance occurred at 4dph, and then swimming performance remained consistent until 6dph (average = 2.17±0.07 cm s^{-1}; 45% increase in U_{crit} compared to previous phase; Figure 5.2). Between 7 and 8 dph, U_{crit} was, on average, 3.04 cm s-1, which still a 40% increase from 4-6dph (Figure 5.2). The largest increase occurred at the time of settlement (9dph), at which point, U_{crit} increased by 118% from 7-8dph U_{crit} values (6.62±0.54 cm s^{-1}; Figure 5.2).

Similar to U_{crit}, $\dot{M}O_2$ also changed over early ontogeny in the anemonefish (Figure 5.3 & 5.4). Estimates of standard metabolic rate (SMR) did not remain constant during the larval phase and changed in distinct ontogenetic phases ($F_{8,68}$ = 61.05, p<0.001; Figure 5.3, table 5.3, 5.4). From 1-4dph, SMR remained constant at 40,997.38±2715.28 mg O_2 kg^{-1} h^{-1} (p=0.7 for all relevant combinations of age and SMR; Figure 5.3). At 5dph, SMR started to decrease with age (Figure 5.3). There was a non-significant 35% decrease in SMR between 1-4dph and 5dph (p=0.06 for all relevant combinations of age and SMR, except when comparing 2dph and 5dph, which were significantly different, p=0.03; Figure 5.3, Table 5.4). There was a further decrease in SMR at 6dph, and then SMR remained constant until settlement at 9dph (p >0.2 for all relevant combinations of age and SMR between 6dph and 9dph; Figure 5.3, Table 5.4). During this last ontogenetic phase (6-9dph), SMR was on average 14,006±628.4 mg O_2 $kg^{-1}h^{-1}$ (Figure 5.3). Despite the 46% decrease in SMR between 5dph and 6-9dph, this decrease was not statistically significant (p >0.03; Figure 5.3). Between the first (1-3dph) and second (6-9dph) phases, SMR decreased by 66% (p<0.001 for all combinations of ages between phases; Figure 5.3, Table 5.4).

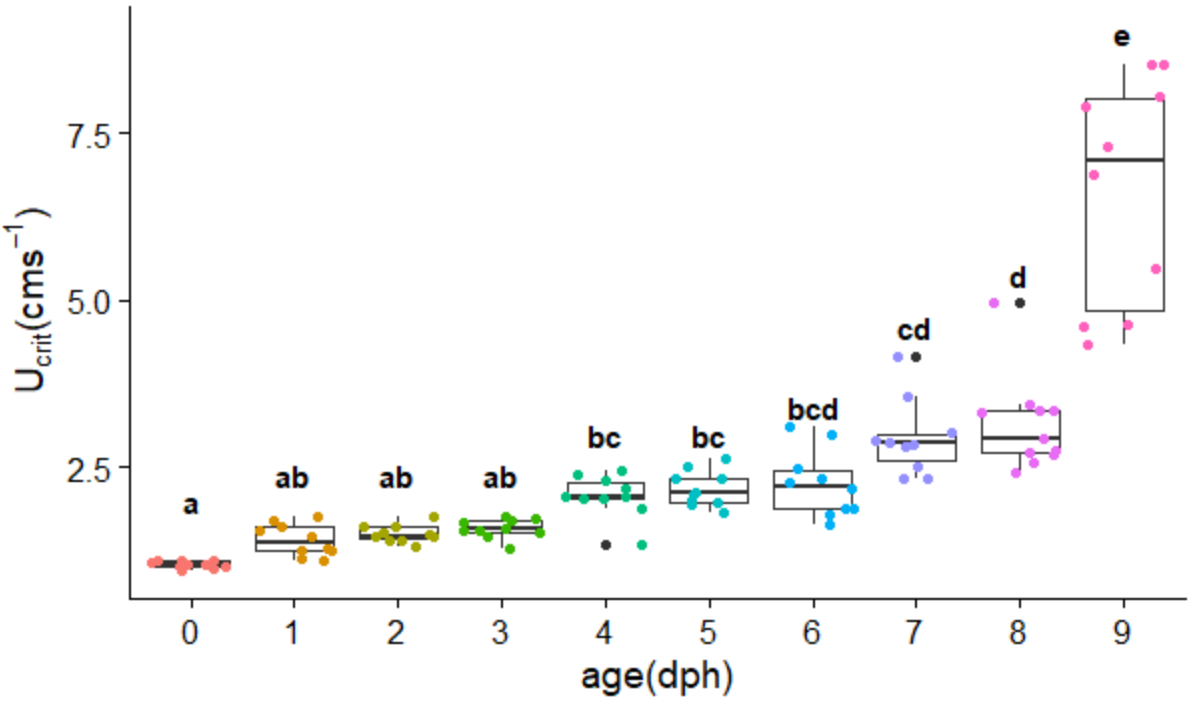

Figure 5.2 The change in critical swimming speed (U_{crit}; cm s^{-1}) of larval cinnamon anemonefish (*Amphiprion melanopus*) over early ontogeny (age; days post hatch; dph). Boxplots show median and interquartile ranges. Daily U_{crit} estimates were taken from time at hatch (0dph) until time of settlement (9dph) (n=10 per developmental age). Different lower-case letters represent statistical differences between groups (α=0.05).

Table 5.1 Model summary of the relationship between critical swimming speed (U_{crit}) and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model= (Ucrit~age, data)

	Df	**Sum sq**	**Mean sq**	**F**	**p**
Age	9	231.4	25.7	61.05	<0.0001
Residuals	91	38.33	0.42		

Table 5.2 Pairwise comparison, using Tukey's post-hoc test, of the differences between critical swimming speed (U_{crit}) and age (dph)

Combination	**Diff**	**lwr**	**upr**	**p-value**
1dph - 0dph	0.373	-0.568	1.31	0.954
2dph - 0dph	0.465	-0.476	1.41	0.843
3dph – 0dph	0.54	-0.401	1.48	0.695
4dph – 0dph	1.03	0.0889	1.97	0.0206
5dph – 0dph	1.13	0.193	2.08	0.00657
6dph – 0dph	1.21	0.273	2.15	0.00253
7dph – 0dph	1.9	0.95	2.84	0.000000002
8dph – 0dph	2.1	1.18	3.016	0.000000001
9dph – 0dph	5.58	4.64	6.52	0.000000001
2dph – 1dph	0.0919	-0.85	1.033	0.999
3dph – 1dph	0.166	-0.775	1.11	0.999
4dph – 1dph	0.657	-0.285	1.6	0.422
5dph – 1dph	0.76	-0.181	1.7	0.223
6dph – 1 dph	0.841	-0.1002	1.78	0.121
7dph – 1dph	1.52	0.58	1.46	0.00000434
8dph – 1dph	1.72	0.803	2.64	0.0000013
9dph – 1dph	5.21	4.27	6.15	0.0000000001
3dph – 2dph	0.0745	-0.867	1.016	0.999
4dph – 2dph	0.565	-0.376	1.51	0.637
5dph – 2dph	0.669	-0.273	1.61	0.396
6dph – 2dph	0.749	-0.192	1.69	0.241
7dph – 2dph	1.43	0.489	2.37	0.000156
8dph – 2dph	1.63	0.71	2.55	0.0000052
9dph – 2dph	5.12	4.18	6.06	0.0000000001
4dph – 3dph	0.49	-0.451	1.43	0.798
5dph – 3dph	0.594	-0.347	1.53	0.568
6dph – 3dph	0.675	-0.267	1.61	0.384
7dph – 3dph	1.36	0.414	2.3	0.000424
8dph – 3dph	1.56	0.636	2.48	0.0000158
9dph – 3dph	5.04	4.1	5.98	0.0000000001
5dph – 4dph	0.104	-0.837	1.05	0.999
6dph – 4dph	0.184	-0.757	1.13	0.999
7dph – 4dph	0.865	-0.0762	1.806	0.0993
8dph – 4dph	1.07	0.146	1.99	0.0107
9dph - 4dph	4.55	3.61	5.5	0.00000000001
6dph – 5dph	0.0805	-0.861	1.02	0.999
7dph – 5dph	0.761	-0.18	1.7	0.222
8dph – 5dph	0.961	0.0419	1.88	0.0329
9dph – 5dph	4.45	3.51	5.39	0.00000000001

7dph – 6dph	0.681	-0.261	1.62	0.371
8dph – 6dph	0.881	-0.0386	1.8	0.072
9dph – 6dph	4.37	3.42	5.31	0.00000000001
8dph – 7dph	0.2	-0.719	1.12	0.999
9dph – 7dph	3.69	2.74	4.63	0.00000000001
9dph – 8dph	3.49	2.57	4.41	0.00000000001

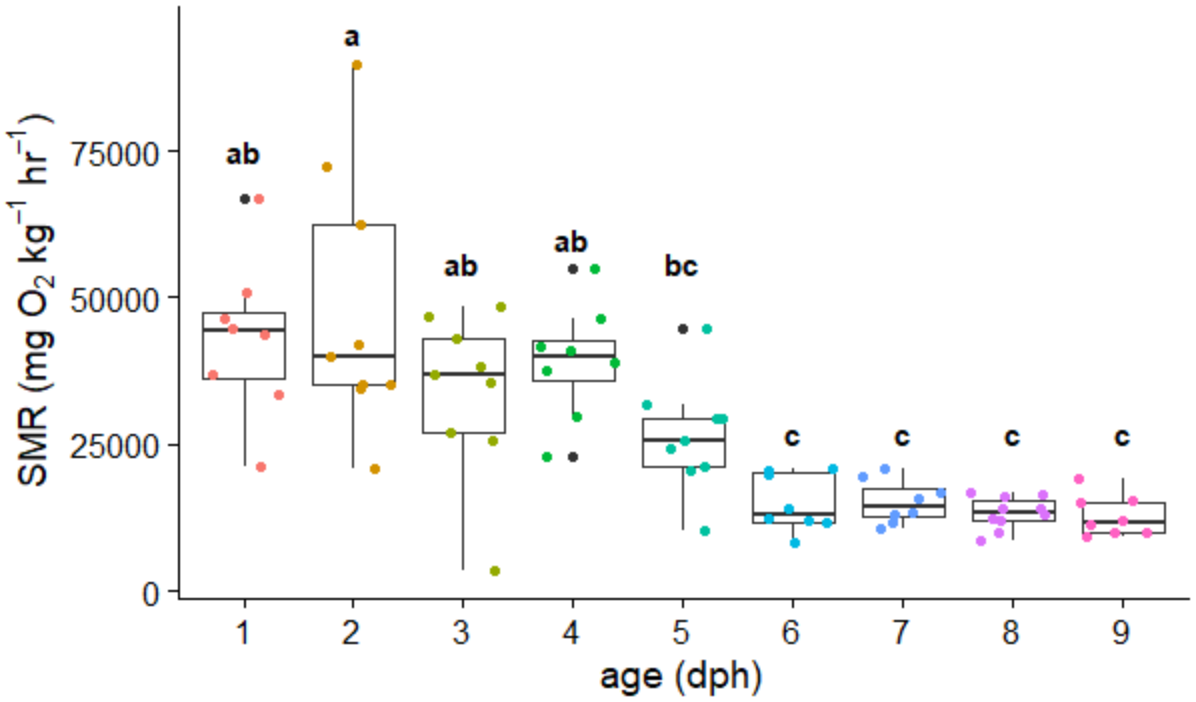

Figure 5.3 The change in standard metabolic rate (SMR; mg O_2 kg^{-1} hr^{-1}) of larval cinnamon anemonefish (*Amphiprion melanopus*) over early ontogeny (age; days post hatch; dph). Boxplots show median and interquartile ranges. SMR estimates were taken daily from time at hatch (0dph) until time of settlement (9dph) (n=8-10 per developmental age) during a U_{crit} test. Different lower-case letters represent statistical differences between groups (α=0.05).

Table 5.3 Model summary of the relationship between standard metabolic rate (SMR) and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(SMR~age, data)

	df	**Sum sq**	**Mean sq**	**F**	**p**
Age	8	1.3e+10	1.7e+9	13.49	<0.0001
Residuals	68	8.4e+9	1.2e+8		

Table 5.4 Pairwise comparison, using Tukey's post-hoc test, of the differences between standard metabolic rate (SMR) and age (dph)

Comparisons	**Diff**	**Lwr**	**upr**	**P**
2dph – 1dph	4891.9	-12439.9	22223.7	0.992
3dph – 1dph	-9157.6	-26489.4	8174.3	0.749
4dph – 1dph	-3912	-21746	13922.3	0.999
5dph – 1dph	-16703.3	-34035	628.5	0.0676
6dph – 1dph	-28169	-46004	-10335.5	0.000114
7dph – 1dph	-27825.4	-45659.7	-9991.1	0.000143
9dph – 1dph	-30263.1	-48097.4	-12428.8	0.00000272
3dph – 2dph	-14049	-30863.8	2764.9	0.176
4dph – 2dph	-8803.9	-26135.7	8527.9	0.786
5dph – 2dph	-21595.2	-38409.5	-4780.9	0.00324
6dph – 2dph	-33061.6	-50393.4	-15729.8	0.0000019
7dph – 2dph	-32717.3	-50049.1	-15385.5	0.0000024
8dph – 2dph	-34621.5	-51010.1	-18232.9	0.0000001
9dph – 2dph	-35155	-52486.8	-17823.2	0.0000004
4dph – 3dph	5245.6	-12086.3	22577.4	0.987
5dph – 3dph	-7545.8	-24360.1	9268.5	0.879
6dph – 3dph	-19012.2	-36344	-1680.4	0.021
7dph – 3dph	-18667.8	-35999.6	-1336	0.025
8dph – 3dph	-20572	-36960.6	-4183.5	0.0044
9dph – 3dph	-21105.6	-38437.4	-3773.8	0.00649
5dph – 4dph	-12791.3	-30123.1	4540.5	0.32
6dph – 4dph	-24257.8	-42092	-6423.5	0.00143
7dph – 4dph	-23913.4	-41747.7	-6079.1	0.00177
8dph – 4dph	-25817.6	-42736.7	-8898.5	0.000215
9dph – 4dph	-26351.1	-44185	-8516.8	0.000378
6dph – 5dph	-11466.4	-28798.2	5865.4	0.469
7dph – 5dph	-11122	-28453.9	6209.8	0.511
8dph – 5dph	-13026.3	-29414.8	3362.3	0.229
9dph – 5dph	-13559.8	-30891.6	3772	0.247
7dph – 6dph	344.375	-17489.9	18178.7	1.0
8dph – 6dph	-1559.9	-18478.9	15359.2	0.999
9dph – 6dph	-2093.4	-19927.7	15740.9	0.999
8dph – 7dph	-1904.2	-18823.3	15014.9	0.999
9dph – 7dph	-2437.8	-20272	15396.5	0.999
9dph – 8dph	-533.5	-17452.6	16385.6	1.0

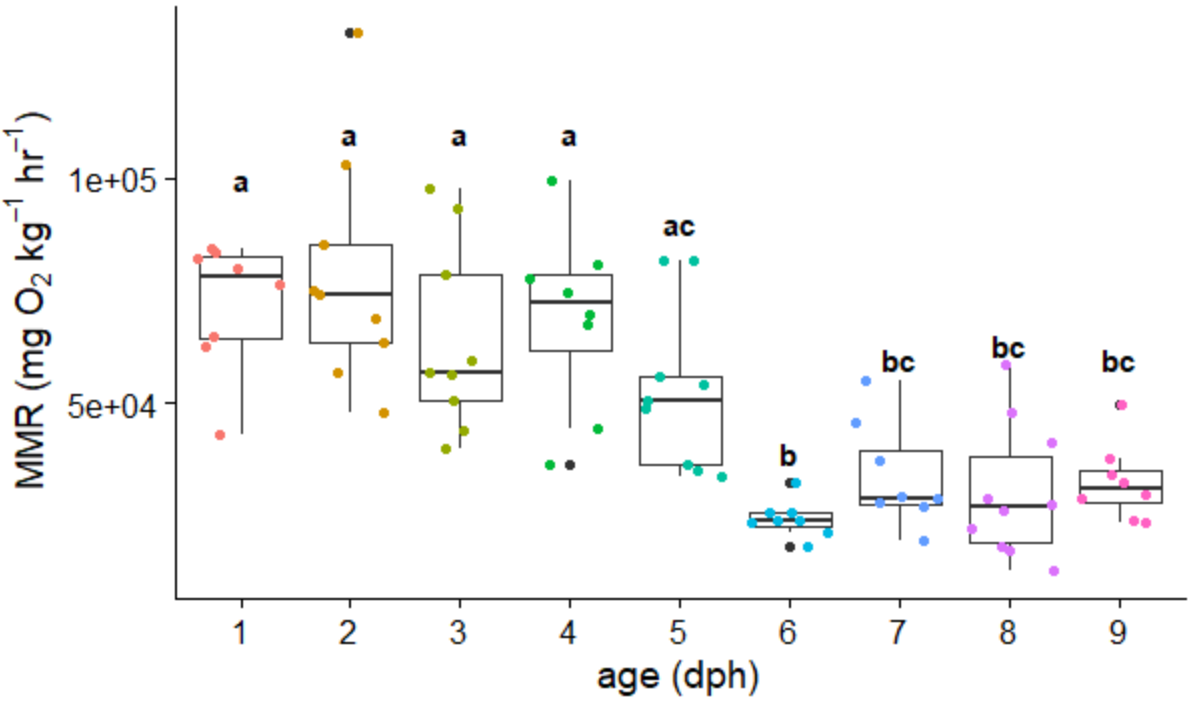

Figure 5.4 The change in maximum metabolic rate (MMR; mg O_2 kg^{-1} hr^{-1}) of larval cinnamon anemonefish (*Amphiprion melanopus*) over early ontogeny (age; days post hatch; dph). Boxplots show median and interquartile ranges. MMR estimates were taken daily from time at hatch (0dph) until time of settlement (9dph) (n=8-10 per developmental age) during a U_{crit} test. Different lower-case letters represent statistical differences between groups (α=0.05).

Table 5.5 Model summary of the relationship between maximum metabolic rate (MMR) and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(MMR~age, data)

	Df	**Sum Sq**	**Mean Sq**	**F**	**p**
Age	8	2.9e+10	3.7e+9	13.2	(<0.0001
Residuals	68	1.9e+10	2.8e+8		

Table 5.6 Pairwise comparison, using Tukey's post-hoc test, of the differences between maximum metabolic rate (MMR) and age (dph).

Comparisons	**Diff**	**lwr**	**Upr**	**P**
2dph – 1dph	6359	-19833.3	32551.3	0.997
3dph – 1dph	-8149.2	-34341.5	18043.1	0.985
4dph – 1dph	-3352.4	-30304	23599.2	0.999
5dph – 1dph	-19091	-45283.3	7101.3	0.336
6dph – 1dph	-48005	-74956.7	-21053.4	0.0000095
7dph – 1ph	-38490.2	-65441.8	-11538.6	0.0000667
8dph – 1dph	-42266.4	-67835	-16697.9	0.0000466
9dph – 1dph	-39755.6	-66707.2	-12803.9	0.000389
3dph – 2dph	-14508.2	-39918.4	10902.1	0.663
4dph – 2dph	-9711.4	-35903.7	16480.9	0.956
5dph – 2dph	-25449.9	-50860.2	-39.7	0.0493
6dh – 2dph	-54364	-80556.3	-28171.7	0.0000002
7dph – 2dph	-44849.2	-71041.5	-18656.9	0.0000225
8dph – 2dph	-48625.4	-73392.3	-23858.6	0.000009
9dph – 2dph	-46114.5	-72306.8	-19922.3	0.0000123
4dph – 3dph	4796.8	-21395.5	30989.1	0.999
5dph – 3dph	-10941.8	-36352.1	14468.4	0.901
6dph – 3dph	-39855.8	-66048.1	-13663.6	0.000226
7dph – 3dph	-30341	-56533.3	-4148.7	0.0117
8dph – 3dph	-34117.2	-58884.1	-9350.4	0.00118
9dph – 3dph	-31606.4	-57798.7	-5414.1	0.00725
5dph – 4dph	-15738.6	-41930.9	10453.7	0.599
6dph – 4dph	-44652.6	-71604.3	-17700.9	0.0000445
7dph – 4dph	-35137.8	-62089.5	-8186.2	0.00264
8dph – 4dph	-38914	-64482.6	-13345.5	0.000225
9dph – 4dph	-36403.2	-63354.8	-9451.5	0.00158
6dph – 5dph	-28914	-55106.3	-2721.7	0.0198
7dph – 5dph	-19399.2	-45591.5	6793.1	0.315
8dph – 5dph	-23175.4	-47942.3	1591.4	0.0845
9dph – 5dph	-20664.6	-46856.9	5527.7	0.238
7dph – 6dph	9514.8	-17436.8	36466.5	0.967
8dph – 6dph	5738.6	-19829.9	31307.2	0.998
9dph – 6dph	8249.5	-18702.2	35201.1	0.986
8dph – 7dph	-3776.2	-29344.8	21792.3	0.999
9dph – 7dph	-1265.4	-28217	25686.3	1.0
9dph – 8dph	2510.9	-23057.7	28079.4	0.999

Changes in maximum metabolic rate (MMR) estimates showed a similar pattern as SMR (Figure 5.4). Estimates of MMR for anemonefish between 1dph and 4dph were statistically similar ($p>0.7$ for all relevant combinations of age and MMR; Figure 5.4, Table 5.5, 5.6). A 25% decrease in MMR occurred between 4dph and 5dph, and similar to SMR, this decrease was also not significant ($F_{8,68} = 13.2$, $p > 0.05$ for all relevant combinations of MMR and age; Figure 5.4, Table 5.6). There was a significant decrease in MMR between 5dph and 6dph ($p=0.01$) but an increase in MMR at 7dph which remained consistent until settlement at 9dph (mean MMR from 6-9dph = 29,969.1±1,871.7 mg O_2 $kg^{-1}h^{-1}$; Figure 5.4, Table 5.6). Similar to SMR, MMR significantly decreased by 58% between the first (1-4dph) and second phase (6-9dph) ($p<0.001$ for all combinations of ages between phases; Figure 5.4, Table 5.6).

Both absolute (MMR-SMR; $F_{8,68} = 2.54$, $p=0.06$, Table 5.7, 5.8) and factorial (MMR SMR^{-1}; $F_{8,68} = 0.69$, $p=0.69$, Table 5.9, 5.10) aerobic scope remained constant during early ontogeny (Figure 5.5). Mean absolute aerobic scope was 23344.4±1693.7 mg O_2 $kg^{-1}h^{-1}$ (Figure 5.5a), and mean factorial aerobic scope was 2.2±0.18 mg O2 $kg^{-1}h^{-1}$ (Figure 5.5b).

Haemoglobin gene expression increases throughout the larval phase of *A. melaopus* (Fig 5.6, Table 5.11, 5.13). Expression of embryonic and adult haemoglobin is highest during the larval stage at 9dph (Figure 5.6), and both genes are significantly higher than earlier ages, respectively (Table 5.12, 5.14). Adult and embryonic haemoglobin gene expression increases by 966% and 21232%, respectively, from 4 to 9dph (Fig 5.6). Following a similar patter to haemoglobin, the gene expression myoglobin (Table 5.15), neuroglobin (Table 5.17), and cytoglobin (Table 5.19) increases over the larval period, with the highest expression at 9dph (Fig 5.7). Myoglobin gene expression increases by 3187% from 4 to 9dph, neuroglobin gene expression increases by 41% from 4 to 9dph, and cytoglobin gene expression increases by 44% from 4 to 9dph (Fig 5.7).

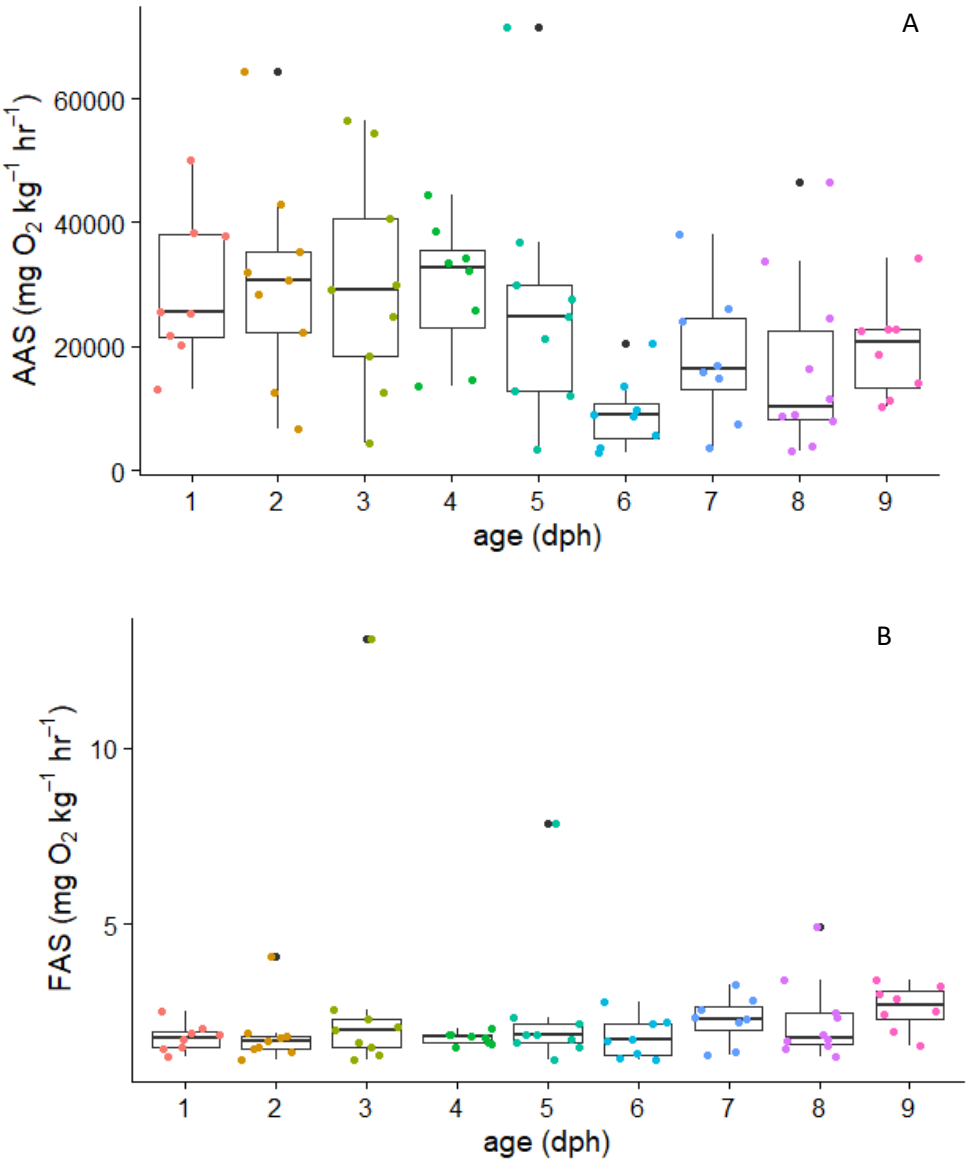

Figure 5.5 The change in a) absolute and b) and factorial aerobic scope (AAS and FAS, respectively; mg O_2 kg^{-1} hr^{-1}) of larval cinnamon anemonefish (*Amphiprion melanopus*) over early ontogeny (age; days post hatch; dph). Boxplots show median and interquartile ranges. Aerobic scope was calculated daily from time at hatch (0dph) until time of settlement (9dph) (n=10 per developmental age) during a U_{crit} test. Different lower-case letters represent statistical differences between groups (α=0.05).

Table 5.7 Model summary of the relationship between absolute aerobic scope (AAS) and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(AAS~age, data)

	df	**Sum sq**	**Mean sq**	**F**	**p**
Age	8	3.9e+9	4.9e8	2.5	0.017
Residuals	68	1.3e10	1.9e8		

Table 5.8 Pairwise comparison, using Tukey's post-hoc test, of the differences absolute aerobic scope (AAS) and age (dph).

Comparison	**Diff**	**Lwr**	**Upr**	**P**
2dph – 1dph	1467.1	-20131.3	23065.5	0.999
3dph – 1dph	1008.4	-20590	22606.8	1.0
4dph – 1dph	559.6	-21664.9	22784.2	1.0
5dph – 1dph	-2387.7	-23986.1	19210.7	0.999
6dph – 1dph	-19835.3	-42059.8	2389.3	0.118
7dph – 1dph	-10664.8	-32889.4	11559.7	0.834
8dph-1dph	-12536.8	-33620.9	8547.2	0.613
9dph – 1dph	-9492.4	-31717	12732.1	0.905
3dph – 2dph	-458.7	-21412.2	20494.8	1.0
4dph – 2dph	-907.5	-22505.9	20690.9	1.0
5dph – 2dph	-3854.8	-24808.3	17098.8	0.999
6dph – 2dph	-21302.4	-42900.8	296	0.0561
7dph – 2dph	-12131.9	-33730.3	9466.5	0.682
8dph – 2dph	-14003.9	-34426.9	6419	0.419
9dph – 2dph	-10959.5	-32557.9	10638.9	0.787
4dph – 3dph	-448.8	-22047.2	21149.6	1.0
5dph – 3dph	-3396	-24349.6	17557.5	0.999
6dph – 3dph	-20843.6	-42442	754.7	0.0668
7dph – 3dph	-11673.2	-33271	9925.2	0.725
8dph – 3dph	-13545.2	-33968.2	6877.8	0.466
9dph – 3dph	-10500.8	-32099.2	11097.6	0.824
5dph – 4dph	-2947.3	-24545.7	18651.1	0.999
6dph – 4dph	-20394.9	-42619.4	1829.7	0.0972
7dph – 4dph	-11224.4	-33448.9	11000.1	0.791
8dph – 4dph	-13096.4	-34180.5	7987.6	0.555
9dph – 4dph	-10052	-32276.6	12172.5	0.874
6dph – 5dph	-17447.6	-39045.9	4150.8	0.211
7dph – 5dph	-8277.2	-29875.6	13321.2	0.947
8dph – 5dph	-10149	-30572.1	10273.8	0.806
9dph – 5dph	-7104.8	-28703.2	14493.6	0.979
7dph – 6dph	9170.4	-13054.1	31395	0.921
8dph – 6dph	7298.4	-13785.6	28382.5	0.97
9dph – 6dph	10342.8	-11881.7	32567.4	0.856
8dph – 7dph	-1872	-22956.1	19212.1	0.999
9dph – 7dph	1172.4	-21052.2	23396.9	1.0
9dph – 8dph	3044.4	-18039.7	24128.5	0.999

Table 5.9 Model summary of the relationship between factorial aerobic scope (AAS) and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(FAS~age, data)

	df	**Sum sq**	**Mean sq**	**F**	**p**
Age	8	14.4	1.8	0.69	0.698
Residuals	68	177	2.6		

Table 5.10 Pairwise comparison, using Tukey's post-hoc test, of the differences factorial aerobic scope (FAS) and age (dph).

Comparison	**Diff**	**Lwr**	**Upr**	**P**
2dph – 1dph	0.0817	-2.43	2.59	1.0
3dph – 1dph	1.29	-1.22	3.8	0.775
4dph – 1dph	-0.0171	-2.6	2.57	1.0
5dph – 1dph	0.681	-1.83	3.19	0.993
6dph – 1 dph	0.00265	-2.58	2.59	1.0
7dph – 1dph	0.505	-2.08	3.09	0.999
8dph – 1dph	0.497	-1.95	2.95	0.999
9dph – 1dph	0.856	-1.73	3.44	0.977
3dph – 2dph	1.21	-1.23	3.64	0.806
4dph – 2dph	-0.0987	-2.61	2.41	1.0
5dph – 2dph	0.6	-1.84	3.04	0.997
6dph – 2dph	-0.079	-2.59	2.43	1.0
7dph – 2dph	0.423	-2.09	2.93	0.999
8dph – 2dph	0.415	-1.96	2.79	0.999
9dph – 2dph	0.774	-1.74	3.29	0.985
4dph - 3dph	-1.31	-3.82	1.2	0.762
5dph – 3dph	-0.61	-3.05	1.83	0.996
6dph – 3dph	-1.29	-3.8	1.22	0.777
7dph – 3dph	-0.787	-3.3	1.72	0.984
8dph – 3dph	-0.794	-3.17	1.58	0.976
9dph – 3dph	-0.435	-2.95	2.08	0.999
5dph – 4dph	0.698	-1.81	3.21	0.992
6dph – 4dph	0.0197	-2.56	2.6	1.0
7dph – 4dph	0.522	-2.06	3.11	0.999
8dph – 4dph	0.514	-1.94	2.97	0.999
9dph – 4dph	0.873	-1.71	3.46	0.975
6dph – 5dph	-0.678	-3.19	1.83	0.994
7dph – 5dph	-0.176	-2.69	2.33	0.999
8dph – 5dph	-0.184	-2.56	2.19	0.999
9dph – 5dph	0.175	-2.34	2.69	0.999
7dph – 6dph	0.502	-2.08	3.09	0.999
8dph – 6dph	0.494	-1.96	2.94	0.999
9dph – 6dph	0.853	-1.73	3.43	0.978
8dph – 7dph	-0.00773	-2.46	2.44	1.0
9dph – 7dph	0.351	-2.23	2.93	0.999
9dph – 8dph	0.359	-2.09	2.81	0.999

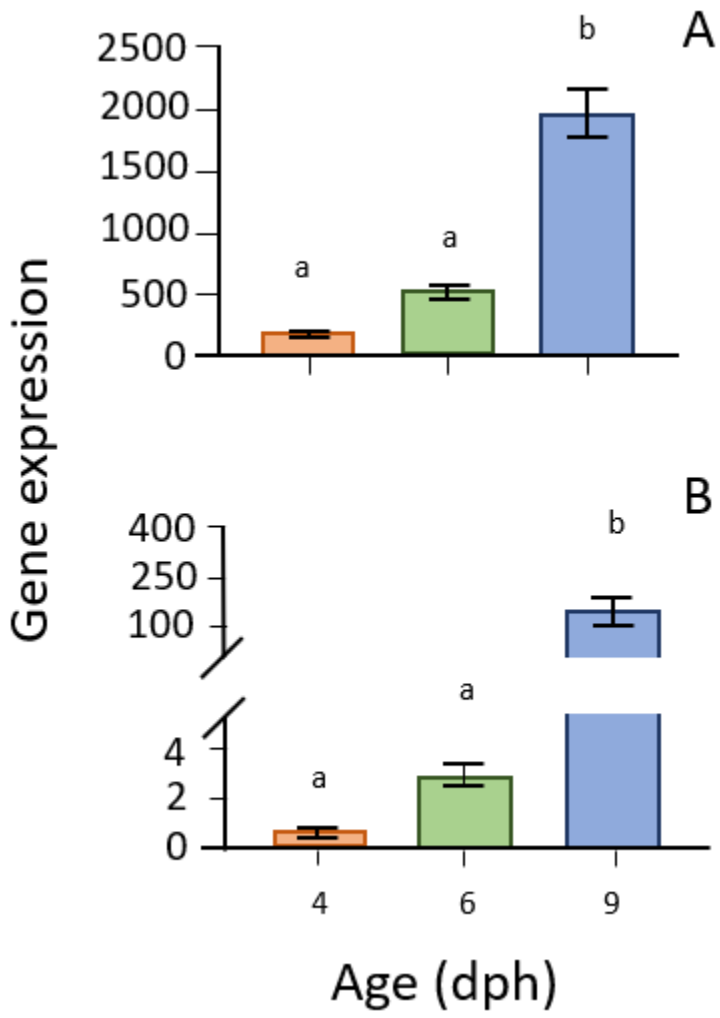

Figure 5.6 Average gene expression (± standard error) of A) adult haemoglobin and B) embryonic haemoglobin of larval cinnamon anemonefish (*Amphiprion melanopus*) at 4, 6, and 9 days post hatch (dph). Different lower-case letters represent statistical differences between groups (α=0.05).

Table 5.11 Model summary of the relationship between adult haemoglobin (Hbb) gene expression and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(Hbadult~age, data)

	Df	**Sum sq**	**Mean sq**	**F**	**P**
Age	2	1e+7	5.2e+6	66.5	<0.0001
Residuals	15	1.2e+6	7.9e+6		

Table 5.12 Pairwise comparison, using Tukey's post-hoc test, of the differences of gene expression of adult haemoglobin (Hbb) and age (dph).

	diff	**Lwr**	**Upr**	**p**
6dph-4dph	354.9	-66.9	776.8	0.106
9dph-4dph	1770.3	1348.5	2192.2	<0.0001
9dph-6dph	1415.4	993.5	1837.3	<0.0001

Table 5.13 Model summary of the relationship between embryonic haemoglobin (Hbe) gene expression and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(hbe~age, data)

	Df	**Sum sq**	**Mean sq**	**F**	**p**
Age	2	9.4e+4	4.7e+4	14.9	0.00027
Residuals	15	4.7e+4	3128		

Table 5.14 Pairwise comparison, using Tukey's post-hoc test, of the differences of gene expression of embryonic haemoglobin (Hbe) and age (dph).

	Diff	**Lwr**	**Upr**	**p**
6dph-4dph	2.12	-81.8	86	0.99
9dph-4dph	154.1	70.3	238	0.00068
9dph-6dph	152	68.1	235.9	0.00077

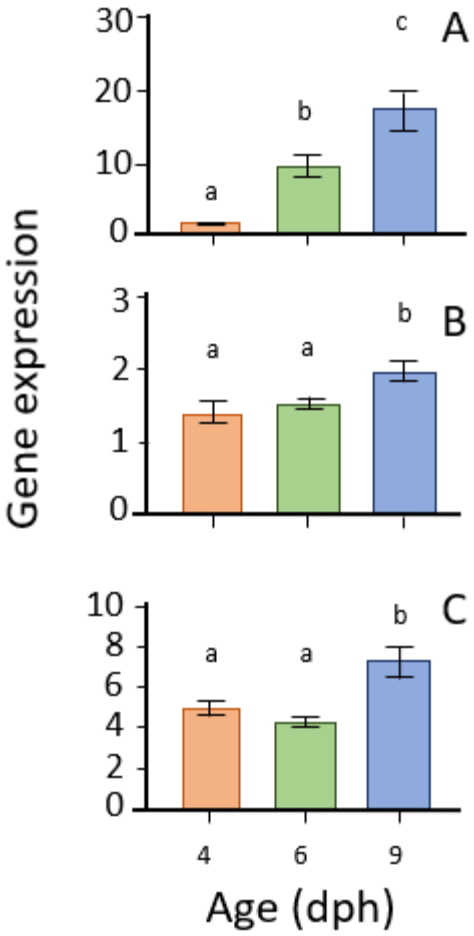

Figure 5.7 Average (± standard error) gene expression of A) myoglobin, B) neuroglobin, and C) cytoglobin of larval cinnamon anemonefish (*Amphiprion melanopus*) at 4, 6, and 9 days post hatch (dph). Different lower-case letters represent statistical differences between groups (α=0.05).

Table 5.15 Model summary of the relationship between myoglobin (Myg) gene expression and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(Myg~age, data)

	df	**Sum sq**	**Mean sq**	**F**	**p**
Age	2	832.7	416.3	21.6	<0.0001
Residuals	15	289.2	19.3		

Table 5.16 Pairwise comparison, using Tukey's post-hoc test, of the differences of gene expression of myoglobin and age (dph).

	Diff	**Lwr**	**Upr**	**p**
6dph-4dph	9.1	2.5	15.7	0.0071
9dph-4dph	16.6	10.1	23.2	0.000025
9dph-6dph	7.5	0.95	14.1	0.024

Table 5.17 Model summary of the relationship between neuroglobin (ngb) gene expression and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(ngb~age, data)

	df	**Sum sq**	**Mean sq**	**F**	**p**
Age	2	1.1	0.55	6.3	0.0102
Residuals	15	1.3	0.087		

Table 5.18 Pairwise comparison, using Tukey's post-hoc test, of the differences of gene expression of neuroglobin and age (dph).

	diff	**Lwr**	**Upr**	**p**
6dph-4dph	0.12	-0.32	0.56	0.75
9dph-4dph	0.57	0.13	1.2	0.011
9dph-6dph	0.45	0.0096	0.89	0.044

Table 5.19 Model summary of the relationship between cytoglobin (cyt) gene expression and age (dph) for cinnamon anemonefishes (*Amphiprion melanopus*) over their larval duration (0-9dph).

Model=(cyt~age, data)

	df	**Sum sq**	**Mean sq**	**F**	**p**
Age	2	27.2	13.6	10.3	0.0015
residuals	15	19.7	1.3		

Table 5.20 Pairwise comparison, using Tukey's post-hoc test, of the differences of gene expression of cytoglobin and age (dph).

	Diff	**Lwr**	**Upr**	**p**
6dph-4dph	-0.69	-2.4	1.03	0.56
9dph-4dph	0.47	0.47	3.9	0.012
9dph-6dph	1.2	1.2	4.6	0.0015

5.5 Discussion

The aim of this chapter was to investigate the oxygen uptake rates that support swimming performance of an anemonefish over its entire larval stage, and to determine whether any measurable changes in oxygen uptake are complimented by changes in gene expression for proteins that transport oxygen (e.g., haemoglobin and other globins). This is critical information since oxygen is a prerequisite for life for all fishes. For early life history, oxygen is crucial to physiological functions such as growth, development, and performance. When compared to juvenile and adult life-stages, the larval phase of fishes is characterised by high oxygen uptake rates due to the high costs associated with development (Post & Lee, 1996; Killen et al., 2007). Supporting hypothesis 3, larval cinnamon anemonefishes have exceptionally high metabolic rates, and this is the first study to elicit such metabolic demands under periods of exercise across early ontogeny. I did find increases in expression genes that code for various haemoglobin isoforms and other globins (e.g., myoglobin, cytoglobin and neuroglobin) across ontogeny. This is among the first studies on a tropical fish species to investigate how genes associated with oxygen uptake, oxygen delivery and hypoxia tolerance are differentially expressed during early life phases. Significant changes in expression patterns of genes associated with oxygen delivery and transport (e.g., haemoglobin) as well as those that protect tissues from hypoxia (e.g., myoglobin and neuroglobin) provide critical links between how changes in gene expression may impact both physiological (e.g., swimming and development) and ecological (e.g., finding a suitable reef home) processes. Overall, the overarching correlation between these aforementioned processes supports the 'athletic' high-capacity swimming performance that enables reef fish in the open ocean to swim in order to find and select for a specific and suitable coastal reef home, and contribute to future generations of reef fishes.

5.5.1. Changes in swimming performance over development

Swimming speed generally increases with age and body length throughout ontogeny among fishes and is also highly variable among individuals at any given size or age (Faria et al., 2009; Faria & Goṇalves, 2010). The critical swimming speed (U_{crit}) test is a reliable method to evaluate how swimming speed changes over ontogeny, with increases in U_{crit} with size or age associated with increases in muscle mass as the larvae grow and develop. The larvae of most fish species are not capable of influential swimming until much later in development (**chapter 2**, Downie et al., 2020). For example, European plaice larvae (*Pleuronectes platessa;* swum at 8°C water temperatures) are not capable of swimming beyond 0.5 cm s^{-1} until they are 10dph (Silva et al., 2015), and the sardine (*Sardina pilchardus*; swum at 15°C water temperatures) cannot swim for more than a few minutes at 1 body length sec^{-1} until 20dph and reaches a maximum swimming speed of 9.47 cm s^{-1} at 55dph (Silva et al., 2014). The cinnamon anemonefish, in the current study, are capable of swimming (<2 cm s^{-1}) at hatch, and they increased their

swimming speed daily with age. There was a significant increase in U_{crit} at 9dph, which correlates with time at settlement. This is a critical period in their life history, as the larvae need to find a reef on which to settle so they can successfully recruit to the adult population (Leis, 2006). A significant increase in U_{crit} near the onset of settlement has been found in cinnamon anemonefish in previous studies (Fisher et al., 2000; Illing et al., 2020). Interestingly, a trend of significant increase in swimming with settlement was not found in the orange clownfish (*Amphiprion percula*), a species that follows an analogous developmental pattern to other tropical anemonefish fish species (e.g., Leis, 2007), however, whereby swimming speed increases linearly with size, including upon settlement (Majoris et al., 2019). Previous studies on cinnamon anemonefish larvae found that settlement stage larvae are capable of swimming beyond 20 cm s^{-1} (Fisher et al., 2000; Illing et al 2021), which are much faster swimming speeds than those derived in the current study (<10 cm s^{-1}). This is likely due to differences in U_{crit} protocols and chamber designs. Different speed and time intervals can alter U_{crit} results (Downie & Kieffer, 2017). Indeed, previous studies (e.g., Fisher et al., 2000) used much faster speed intervals (3 BL s^{-1}) and shorter time intervals (2 min) than the current study (1 BL s^{-1} every 20 min); my protocol required longer measurement periods so that changes in water oxygen levels could be accurately measured to estimate SMR and MMR. Additionally, the Blazka-style swimming respirometer used in the current study is more constrictive than a large, multi-lane raceway design of previous studies, which may not allow swimming larvae the same amount of space to perform burst swimming that can allow them to achieve higher swimming speeds. Regardless, cinnamon anemonefishes are capable of swimming upon hatch and develop their swimming ability in daily increments until there is a significant increase in swimming speeds as settlement approaches. High oxygen uptake is critical to support such performance at hatch and across the larval phase, as well as to support basic tissue maintenance and growth.

5.5.2 Changes in oxygen uptake rates over development

Across their entire larval duration, the cinnamon anemonefish used in the current study exhibited the highest oxygen uptake rates (i.e., SMR, MMR, and AAS) of any teleost fish in the published literature to date. Moreover, anemonefish exhibit distinct changes in metabolic demands with age. Although, few studies have measured $\dot{M}O_2$ across the entire larval duration of any teleost fish species (e.g., Killen et al., 2007; Edworthy et al., 2018), to date, seemingly no study has involved swimming respirometry to calculate aerobic scope daily. Therefore, it should be noted early that meaningful differences in $\dot{M}O_2$ between the current and previous studies are difficult, due to such methodological differences (described below) and given that very few studies exist. Regardless, metabolic rate estimates for temperate, demersal species during their larval stage at a similar size (shorthorn sculpin, *Myxocephalus scorpius,* mass = 0.01g experimental water temperature = 3°C; lumpfish *Cyclopterus lumpus*, mass = 0.01g, experimental water

temperature = 11°C) are considerably lower (SMR = 400 and 500 mg O_2 $kg^{-1}hr^{-1}$, respectively; MMR= 800 mg O_2 $kg^{-1}hr^{-1}$ for both species) to the anemonefish studied here, likely due to the significant differences in water temperature (current study, 28°C) and methodology (Killen et al., 2007). However, even at a similar temperature (10°C), European plaice (*Pleuronectes platessa*), a benthic flatfish species, have much higher pre-flexion (2410 mg O2 $kg^{-1}hr^{-1}$) and post-flexion (4560 mg O2 $kg^{-1}hr^{-1}$) $\dot{M}O_2$ measurements than the lumpfish and sculpin, possibly due to the energy required for flatfishes to undergo significant morphological and anatomical modifications during metamorphosis (de Silva & Tytler, 1973; Almatar, 1984). Habitat may also play an integral role, as larvae of the pelagic herring species *Clupea harengus* have much higher pre-flexion (3300 mg O_2 $kg^{-1}hr^{-1}$) and post-flexion (5100 mg O_2 $kg^{-1}hr^{-1}$) $\dot{M}O_2$ levels than those aforementioned species. This suggests the developmental costs of remaining in the pelagic environmental may be different than those required to find a benthic habitat for temperate fishes (Almatar, 1984; Illing et al., 2018). While estimates of SMR and MMR and aerobic scope calculations are highly variable between species and ontogenetic stages, particularly across tropical and temperate latitudes, the high metabolic costs of cinnamon anemonefishes may be associated with how advanced in development these fishes are at hatch.

The vast majority of marine teleosts are pelagic spawners, whereby males release sperm and females release eggs into the water column, thus producing thousands to millions of small eggs. Generally, most fishes that hatch from pelagic eggs are tiny and underdeveloped (**chapter 2**). Upon hatch, most larval fishes rely mainly on cutaneous oxygen uptake until gills develop (reviewed in Downie et al., 2020). However, direct-developing fishes (i.e., species with more parental investment into better developed larvae at hatch, like anemonefishes), hatch with better developed swimming structures (e.g., fins), muscles, and eyes (Roux et al., 2019), as well as functional gills (Prescott et al. revisions submitted). For direct developing fishes, it has been suggested that at hatch the metabolic demands would be much higher in order to provide oxygen to such complex structures (Oikawa et al., 1991). This prediction by Oikawa et al., (1991) is supported by the current study, as both SMR and MMR are highest within the first 4 days post-hatch, suggesting that: i) the metabolic demands associated with basic maintenance and growth are high to enable larvae to support complex tissues from hatch and grow larger to swim more efficiently and reduce predation risk, ii) these demands need to be high, as cinnamon anemonefish have a very short larval duration (9 days) and need to rapidly synthesize tissue before settling onto the reef (as predicted by **chapter 4**), and iii) the high maximum metabolic rates suggest that larvae are not swimming at maximum speeds. The decrease in MMR and SMR around 4-6dph may be due to the increase in the white, less metabolically active, muscle tissue with growth or changes in genes associated with oxygen uptake and delivery (described below). Interestingly, despite these significant

changes in oxygen uptake rates over early ontogeny, aerobic scope was maintained across the entire larval phase.

Aerobic scope is the energy budget for all physiological tasks powered by oxygen (Rummer et al., 2016). Critical functions such as swimming, growth and development that allow an animal to respond to its environment are powered by energy that is derived from the total aerobic scope. Changes in aerobic scope, either due to ontogeny or from external stressors (e.g., temperature), may have ecological consequences for the animal (e.g., less energy available for swimming, responding to stress). For the cinnamon anemonefish, aerobic scope was consistent across the larval phase, suggesting that either overall energetic demands powering all physiological functions remain relatively constant or there is a 'metabolic reshuffling' with some traits/behaviours and tissues taking up more energy later in development at the expense of other taking up less. Cinnamon anemonefishes have a very high absolute aerobic scope throughout their larval phase, likely due to high basic maintenance costs and high metabolic rates during periods of maximum exercise. While this suggests they can direct more oxygen to their tissues than other teleost larvae, their factorial aerobic scope (FAS; the ratio of MMR to SMR), which is an indicator of how much above maintenance levels an animal can raise its metabolic rate (Killen et al., 2007) is low (generally <2) but consistent with previous work on aerobic scope of larval fishes (Killen et al., 2007; Laubenstein et al., 2018). Such a narrow FAS suggests that larval anemonefishes have a limited aerobic capacity outside of their vital physiological functions (i.e., beyond SMR) and may have difficulties "physiologically multi-tasking" when faced with environmental stress (Killen et al., 2007). Therefore, due to their high metabolic costs, larval anemonefishes must optimize their energy budget; otherwise, and as previously suggested, high metabolic costs for maintenance (i.e., SMR) may be disadvantageous under scenarios where food is limiting, as larvae would become easily starved of energy to support basic function (Bochdansky et al., 2005; McLeod et al., 2013). If larvae are exposed to limited or patchy resources under unpredictable environmental conditions, starvation or even death could ensue. Over generations, evolutionary trade-offs could result in sacrifices to larval duration, growth rates, swimming capacity, and other metabolic costs to facilitate settlement on a reef. Future work comparing metabolic demands of direct developing fishes in contrast with broadcast spawners may provide insight into the evolutionary advantages of advanced development at hatch, despite high metabolic costs. Regardless, these notable differences in metabolic rates between the current and previous studies may be a result of several differences in methodology.

Historically, most studies (approximately 95% of 59 studies since 2016) that use oxygen uptake rates as a proxy for metabolic rate on fish larvae report routine metabolic rates (RMR) as a representation of metabolic demands of an un-fasted individual exhibiting normal behaviours without forced exercise

under static respirometry protocols (Peck & Moyano, 2016). Estimates of RMR are within the range of 1000 to 1200 mg O_2 kg^{-1} h^{-1} for settlement-stage larvae of closely related orange clownfish (*Amphiprion percula*; McLeod et al., 2013) and Ambon damselfish (Ambon damselfish (*Pomacentrus amboinensis*; Killen et al., 2014). However, these measurements do not provide an estimate of metabolic demands associated with swimming or allow for aerobic scope to be determined, which are important metrics for reef fishes that have high swimming capabilities and need to find a reef on which to settle. Generally, MMR estimates for larval or juvenile fishes use a short (2 min) chase protocol ((Hess et al., 2017; Killen et al., 2007, 2014; Laubenstein et al., 2018, 2019, 2020), and therefore maximum oxygen uptake rates are likely associated with repaying an oxygen debt from anaerobic burst swimming, versus oxygen consumed from exhaustive exercise (e.g., excess post-exercise oxygen consumption, EPOC; Killen et al., 2007). These methodological differences are important, as there are quantifiable differences in $\dot{M}O_2$ (up to 40%) during activity between chase and U_{crit} protocols; however, differences are species (Rummer et al., 2016), and likely life-stage specific. Additionally, studies measuring $\dot{M}O_2$ on dusky kob (*Argyrosomus japonicus*; Edworthy et al., 2018) and Cape horse mackerel (*Trachurus capensis*; Geist et al., 2013) derive MMR from the highest 5% of $\dot{M}O_2$ values during static respirometry experiments; however, these methods predictably highly underestimate MMR, as these individuals are not under forced exercise conditions. Studies that use forced swimming protocols (e.g., U_{crit}) on fish larvae are few (reviewed in Peck & Moyano, 2016), making results from the current study highly valuable, yet difficult to compare with previous studies using static respirometry methods. The only example that measured oxygen uptake rates during activity of settlement stage reef fishes used a circular chamber that generated a current via a magnetic stir bar (Nilsson et al., 2007a). Oxygen uptake rates during these experiments at the time were noteworthy for being the highest $\dot{M}O_2$ of any teleost fish species (Ambon damselfish, 32.9mg, $\dot{M}O_2$ = 3753 mg kg^{-1} hr^{-1}; black-axil chromis, *Chromis pectoralis*, 19.4mg, $\dot{M}O_2$ = 3250mg O_2 kg^{-1} hr^{-1}); however, these individuals were much larger and represented later ontogenetic stages than the larvae used in the current study.

The high oxygen uptake rates of cinnamon anemonefish larvae are not only attributed to the fact that $\dot{M}O_2$ was measured during forced exercise (versus under static conditions), but may be also be attributed to ontogenetic differences between previous studies. The highest oxygen uptake rates determined in this study occurred during the first 4 days of the larvae's life. The challenges associated with measuring oxygen uptake rates of fish larvae were largely associated with limitations in technology, which have generally limited $\dot{M}O_2$ measurements to later larval stages (Peck & Moyano, 2016). Early respirometry work on larval fishes (1950s to early 2000s) used electrodes that were inaccurate and consumed oxygen during measurements, thus masking oxygen uptake rates of larvae (Peck & Moyano, 2016). Additionally, micro-swimming respirometers were not commonly used, as it was difficult to create

a small swimming chamber that could accurately measure $\dot{M}O_2$ and maintain structural integrity under high flow conditions. These challenges were addressed in the current swimming respirometer design, which allowed $\dot{M}O_2$ measurements early in life history and across the entire larval stage of the cinnamon anemonefish. Fiberoptic dip probes were used in favour of electrodes due to highly accurate measures of minute changes in water oxygen levels and using a variable speed motor allows for slow (<1 cm s^{-1}) increments in water flow, which is especially important for larvae a few days post-hatch. The chamber inserts allowed for drastic reduction in the volume of the respirometer, instead of creating multiple small respirometers, which further facilitated accurate $\dot{M}O_2$ measurements from very small animals during exercise. The significance of these advancements in larval fish respirometry permits investigating metabolic demands during exercise at critical periods of time in early ontogeny that older techniques could not support. Broadening the scope of larval stages (i.e., hatch until settlement) used in my experiments, versus focusing exclusively on older larvae (i.e., post flexion stages), not only highlights the high amount of oxygen required to support vital physiological functions for these fish, but also showed unique patterns in oxygen uptake over early ontogeny. These patterns became the foundation on which I investigated the importance of changes in expression patterns of genes responsible for regulating oxygen uptake and delivery throughout the larval phase.

5.5.3 Changes in expression of genes associated with oxygen delivery and transport proteins

I found several changes in the expression of genes associated with oxygen transport (haemoglobin, Hb), oxygen diffusion into tissues (myoglobin, Mb), and oxygen storage (Mb, cytoglobin Cygb, and neuroglobin, Ngb). My initial hypothesis was that there would be an observable switch in haemoglobin from low to high oxygen affinity, which is essentially the reverse of what has been found in salmonids. Interestingly, both adult and embryonic haemoglobin subunits were highly expressed and increased at the point of settlement, along with other globin groups, such as myoglobin, cytoglobin, and neuroglobin. This may be due to changing oxygen demands as fish larvae increase their swimming performance and transition to an environment where hypoxia tolerance is required.

5.5.3.1 Changes in haemoglobin gene expression over larval development

Over vertebrate development, oxygen demands change (e.g., energy requirements for growth/organogenesis, ontogenetic shifts in habitats with different oxygen levels etc.), and in response, haemoglobins change their binding affinity for oxygen to adjust and respond to such changes (Rohlfing et al 2016). For example, Mozambique tilapia (*Sarothodon mossambicus*) express additional haemoglobins during adult life stages that are not modified by changes in salinity or temperature, thus allowing the fish to exploit a range of habitat conditions (Pérez & Maclean, 1976). During the early life history stages of

teleost fishes (i.e., embryonic and larval stages) Hb-O_2 affinity is optimized to the balance between oxygen loading and unloading in fast growing larval stages, regardless of environmental oxygen levels (Bianchini & Wright, 2013). Embryonic haemoglobins are generally cathodic, characterised by high binding affinities with oxygen, meaning that they bind oxygen even when pH decreases (Brownlie et al., 2003). However, the anemonefish larvae in the current study expressed both embryonic and adult haemoglobins throughout their larval phase. Moreover, gene expression of both embryonic and adult forms was highest at settlement. This pattern may in response to the increased swimming capabilities as the larvae prepares to settle and the need to become hypoxic tolerant upon reaching the reef.

Hypoxia is a common stressor experienced by teleost fishes over their entire life history and is a driver for why many teleost fishes express a range of different haemoglobins with different affinities for oxygen (Brauner & Weber, 1998; Imsland et al., 1997). Many species, such as cichlids (Rutjes et al., 2007) and red drum (*Sciaenops ocellatus*; Pan et al., 2017), switch to haemoglobins with high oxygen affinities in response to hypoxic conditions to prevent oxygen starvation to vital tissues. While a definitive switch in haemoglobin expression was not observed in larval anemonefishes in the current study, the retention of embryonic haemoglobins and their high expression late in the larval period, as seen here, has been observed in other fish species. In zebrafish (*Danio rerio*), embryonic haemoglobin is significantly expressed from 31 hpf (hours post-fertilization) until 54 dpf (days post-fertilization); afterwards, expression is believed to decline and switch completely to adult haemoglobins (Tiedke et al., 2011). Adult haemoglobin gene expression in zebrafish is initiated by 16dpf; although, it is expressed by much less than embryonic haemoglobin (Tiedke et al., 2011). Additionally, gilthead seabream (*Sparus aurata*), a euryhaline and eurythermal species, retains embryonic haemoglobins to secure oxygen delivery to tissues over a wide gradient of habitat conditions (Mania et al., 2019). The high binding affinity of embryonic haemoglobins is of 'emergency' use to ensure oxygen delivery to tissues when blood pH decreases due to hypoxia and intense exercise (Brownlie et al., 2003). It is possible that for anemonefishes, embryonic haemoglobin exhibits the highest expression patterns at settlement (9dph), perhaps the most critical life history stage with respect to swimming performance and challenging environmental conditions. At this developmental stage, swimming performance is at its highest, and therefore oxygen delivery during the acidosis ensued upon exercise would be critical. Moreover, upon transitioning to the reef, anemonefish, and perhaps other coral reef fishes, must prepare for nocturnal hypoxic events (Nilsson et al., 2007a,b). In addition to retaining embryonic haemoglobins, anemonefish in this study may have been differentially expressing other globins across early development as well.

5.5.3.2. Changes in myoglobin gene expression over larval development

Myoglobin is a protein related to haemoglobin but has notable functions for oxygen storage (e.g., during hypoxia events), buffers oxygen levels in tissues when muscle activity increases, and facilitates oxygen diffusion into tissues, particularly during intense exercise and hypoxia (Vlecken et al., 2009; Kanatous & Mammen, 2010). Myoglobin is generally found in skeletal muscle and cardiac tissue and is prevalent during embryonic stages, seemingly to aid in organogenesis and particularly in cardiac tissue (Vlecken et al., 2009). Myoglobin also increases in young animals as locomotor capabilities increase (Garry et al., 1998; Noren et al., 2001). Myoglobin concentration has been strongly correlated with hypoxia tolerance, as many hypoxia tolerant groups of fishes (e.g., cyprinids, such as goldfish and carp, zebrafish, and elephantnose *Gnathonemus petersii*) express high levels of myoglobin (Gallagher & Macqueen, 2017; Nilsson, 1996). The anemonefishes in the current study weakly express myoglobin until 6dph, at which point myoglobin expression increases to the point of settlement (9dph). In contrast, myoglobin is present in zebrafish tissues at hatch, but is not highly expressed until 31 hph (hours post hatch; Tiedke et al., 2011). Given its role in hypoxia tolerance and oxygen diffusion into the tissues, the fact that myoglobin expression is highest at the point where swimming ability in an anemonefish is highest may be key. This may suggest that myoglobin is important for maintaining high swimming performance and perhaps to facilitate oxygen diffusion to swimming muscles. This is also the stage when the larvae are transitioning to the reef, and therefore oxygen storage may be key for successful reef recruitment under nocturnal hypoxic conditions. Several groups of fishes, including sticklebacks (Gasterosteidae) and icefishes (Notothenioidei), do not express myoglobin in organs where it is traditionally expressed, such as cardiac or skeletal muscle tissues, but rather express myoglobin in tissues such as the brain and swim bladder (Daane et al., 2020; Gallagher & Macqueen, 2017; Sidell & O'Brien, 2006). While there may be a clear role for increasing myoglobin expression in preparation for settlement, still other globins, such as cytoglobin and neuroglobin, may be involved.

5.5.3.3. Changes in cytoglobin and neuroglobin gene expression over larval development

Over evolutionary history myoglobin expression in cardiac and skeletal muscles has been lost in a few taxa of fishes (i.e., notably icefishes), and replaced with cytoglobin and neuroglobin (Gallagher & Macqueen, 2017). Neuroglobin is expressed in the cells of the central and peripheral nervous system of vertebrates and supplies oxygen to metabolically demanding neural and endocrine tissue and is also thought to aid survival under hypoxia conditions (Wystub et al., 2004). Cytoglobin is present in connective tissue and has been found to be upregulated during periods of oxygen deprivation as an alternative to myoglobin for oxygen storage (Wystub et al., 2004). In zebrafish, neuroglobin expression follows a similar pattern of expression as myoglobin (Tiedke et al., 2011). In anemonefish larvae, unlike

myoglobin, which is not regulated until at least 6dph, neuroglobin and cytoglobin are expressed in earlier stages, but reach their peak expression at 9dph. However, similar to both haemoglobin and myoglobin, high gene expression of both cytoglobin and neuroglobin at settlement may also help retain oxygen (i.e., via cytoglobin) and provide sufficient oxygen to the brain (i.e., via neuroglobin) during recruitment to the reef and upon preparing for chronic nocturnal hypoxia events.

5.5.3.4. Conclusion

Until this study was conceived, investigating the oxygen uptake rates during periods of exercise over the entire larval stage of a teleost fish species had yet to be accomplished. Modifying the design of current larval swimming respirometry chambers has allowed for precise measurements of larval fishes during periods of exercise, which will open many potential avenues for future research, such as understanding how larval fishes alter oxygen uptake rates in response to anthropogenic and natural stressors (e.g., warming, ocean acidification, etc.). Anemonefishes in the current chapter exhibit high oxygen uptake rates, likely to support complex tissues at hatch, supporting my initial hypothesis. Anemonefishes also exhibit strong swimming performance throughout ontogeny (i.e., especially when compared with temperate fish larvae), particularly at the point of settlement which is when they need to find a suitable reef home. However, oxygen uptake rates follow an interesting pattern; rates significantly decrease around 6dph until settlement. The mechanism driving the change in oxygen uptake over larval ontogeny with gene expression for proteins associated with oxygen delivery, transport and storage is novel and significant for a better understand what drives swimming performance during the larval phase of reef fishes.

Comparing expression of relevant genes (i.e., those coding for oxygen transport) with whole animal performance (i.e., swimming and metabolism) can help reveal the mechanistic link between animal activity and the cellular and molecular processes mediating these functions. This link is still widely unknown (i.e., correlation does not necessarily mean causation), and the conclusions from this chapter will set the groundwork for future studies investigating the link between gene expression with animal performance. For example, future studies could investigate hypoxia tolerance of reef fishes during larval stages to make this link more definitive. Additionally, investigating gene expression during embryonic, larval and adult phases may reveal a more characteristic shift in haemoglobin isoforms, as seen in other fishes like salmonids. Regardless, the patterns in oxygen uptake, gene expression, and swimming performance reveals the link these different levels of biological organization have to one another. For reef fishes, this link is highly important as genetic control over proteins that regulate oxygen uptake (haemoglobin), delivery to tissue (myoglobin), and storage in tissue (neuroglobin and cytoglobin) that supports high growth and swimming performance in the pelagic environment (i.e., high oxygen

uptake rates for dispersal), must switch to enable the larva to become hypoxia tolerant (i.e., lower oxygen uptake rates for successful reef transition). This critical transition period, known as metamorphosis, will be the focus of next chapter of my thesis.

Chapter 6. Changes in Oxygen Uptake Rates and Muscle Mitochondria Volume Densities at Metamorphosis in a Reef Fish

6.1 Summary

The transition larval reef fishes make to a reef at the end of their larval phase in the ocean is known as settlement and marks an important milestone in theirement life history. This ecological transition is coincident with a developmental transition (metamorphosis), which is characterised by changes in colour, morphology and physiology, associated with surviving in two very different environments. Some studies have found notable decreases in swimming performance as reef fish larvae metamorphose and this may relate in part to differences in the oxygen environment experienced. The changes in physiology that complement the pelagic to reef transition are poorly known. Here I investigated the changes in oxygen uptake rates and oxidative capacity of tissues (i.e., muscle mitochondria volume densities) that limit swimming after metamorphosis, in a common reef damselfish (*Pomacentrus amboinensis*). Using swimming respirometry techniques, I found that swimming speed decreased by 40% within 72 h of metamorphosis, however, there were no changes in oxygen uptake rates despite this decrease in swimming ability. Muscle mitochondria volume densities increased by 20% within 24 h of metamorphosis, before dropping back down 72 h later. This increase in muscle mitochondria volume density was also complemented by a decrease in Cost of Transport, which allowed fish to swim more efficiently. These rapid changes in swimming behaviour and oxidative properties of muscle tissues underscore the dramatic changes in major selective pressures that must occur over this critical transition.

Associated Publication

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Data availability

Data present in this chapter are available from the Research Data Repository (Tropical Data Hub) at James Cook University. DOI: https://doi.org/10.25903/j9rx-7a14

6.2 Introduction

Metamorphosis in the animal kingdom

Metamorphosis is a biological process that has captured the interest of scientists for decades, specifically for its relevance across many subdisciplines, including developmental biology, physiology, cell biology, ecology, and evolution (Bishop et al., 2006). In general, metamorphosis is defined as an ontogenetic change in morphology, physiology, and ecology (e.g., upon dispersal to a new habitat) (Bishop et al., 2006; Vigliola & Harmelin-Vivien, 2001), and thus represents a link between development of appropriate structures to respond to and maximize survival in a new environment (Balon, 1985; McCormick, 1993; McCormick et al., 2002). Consequently, metamorphosis is widespread across the Animal Kingdom, and thus the exact definition is highly debated. Specifically, metamorphosis has appeared independently across several chordate lineages (e.g., anurans, Actinopterygian fishes) (Paris & Laudet, 2008); however, the mechanisms underpinning metamorphosis in each taxa are highly species specific. Much of the current knowledge based on vertebrate metamorphosis has focused on anurans, specifically the bullfrog (*Rana catesbeiana*), and a small number of fishes.

Patterns characterising metamorphosis events for marine fishes

Most of the current knowledge base involving metamorphosis of teleost fishes is based on marine species. Metamorphosis in most marine fishes involves a change in habitat; generally, a pelagic larva descends to the benthos and changes its morphology and physiology to be best suited for a demersal lifestyle (Lecchini et al., 2007). Therefore, many species of marine fishes are classified under the definition of 'saltatory ontogeny' (Balon, 1985, 1990), whereby life stages are associated with ontogenetic changes in morphology and habitat use. This transition from the pelagic environment is mediated by environmental cues and hormones (McCormick & Molony, 1992; McCormick, 1993; Bishop et al., 2006). Flatfishes (Pleuronectiformes) have received the most research attention due to their unique shift from a symmetrical, free swimming larva to a benthic, asymmetrical (i.e., eyes on one side of the head) juvenile/adult (Paris & Laudet, 2008). However, metamorphosis is a crucial transition event for most tropical, coral reef fishes as well (McCormick & Molony, 1992; McCormick, 1993; McCormick et al., 2002).

At the end of their pelagic larval duration (PLD), reef fish larvae transition to a benthic reef habitat, representing a shift in environmental conditions (e.g., light, temperature, water current velocity etc.), known as settlement (McCormick & Molony, 1992). Larvae facilitate this transition, known as 'settlement transition' (McCormick & Makey, 1997), from a pelagic to a benthic lifestyle, through many morphological (e.g., pigment changes; McCormick et al., 2002), anatomical (e.g., appearance of dorsal

and pectoral spines; McCormick et al., 2002), and physiological changes (e.g., goatfish *Upeneus tragula* develop barbels for chemoreception and prey manipulation; McCormick, 1993). These unique changes define metamorphosis in coral reef fishes and must occur rapidly (i.e., often <24 hrs). Differences in the nature and selectivity of predation between pelagic and reef habitats has likely led to the rapid change in morphology, physiology, and behaviour associated with settlement. While much of the research on coral reef fish metamorphosis has focused on morphological/anatomical changes and the temporal scale over which they occur, there is great interest in how performance changes when coral reef fishes settle onto the reef.

There have been notable observations of the significant decline in swimming performance of coral reef fishes upon settlement. Decrease in settlement was first reported by Stobutzki & Bellwood, (1994); they found that recently metamorphosed pomacentrid juveniles decreased their critical swimming speed (i.e., maximum swimming speed that can be maintained primarily aerobically; U_{crit}) by 31% when compared to pre-settled larval conspecifics. Similarly, the median U_{crit} of fivestripe wrasse (*Thalassoma quinquvittatum*) decreased by 73% within 24 hours and 83% by 48 hours upon settlement (Leis et al., 2011). In contrast, the sub-tropical gilt-head bream (*Sparus auratus*) increased U_{crit} by 28-45% after metamorphosis (Koumoundouros et al., 2009). Since swimming performance is closely related to oxygen uptake ($\dot{M}O_2$), the decrease in Maximum Metabolic Rate (MMR; see chapter 5 for definition) observed in metamorphosed black-axil chromis (*Chromis atripectoralis*) and Ambon damselfish (*Pomacentrus amboinensis*) (Nilsson et al., 2007b) supports the decrease in U_{crit} observed in the previous studies. The decrease in performance observed in these few reef-associated species has been hypothesized to occur in response to the environment in which reef fish larvae settle. Coral reefs are structurally complex habitats; here, fishes do not require fast speeds for navigation as they would in the pelagic environment (Korsmeyer et al., 2002). Additionally, reef fish larvae have high oxygen uptake rates to support swimming performance during their pelagic phase (**chapter 5**). Some habitats within coral reefs can become hypoxic at night due to coral and plant respiration (Nilsson et al., 2007a,b). Generally, hypoxia tolerant fishes have low oxygen uptake rates (Nilsson et al., 2007a,b). Therefore, many coral reef fishes must make a rapid change in their physiology to adjust to these conditions upon reaching a reef, but it is generally unknown how they do this.

Fishes that are active as larvae and then settle to a benthic/less active lifestyle as a juvenile have been shown to exhibit changes in many physiological characteristics over settlement, such as decreased swimming performance. However, much of this work has focused on temperate or polar species (Johnston & Camm, 1987; Moyes et al., 1992; Finn et al., 2002). These past studies highlight that a decrease in swimming performance is generally coincident with a decrease in mitochondrial volume densities (V_{mt}) in

swimming musculature (Egginton, 1986; Johnston & Camm, 1987; Finn et al., 2002). Mitochondria are the organelles responsible for biochemical processes associated with respiration and are a source for ATP (adenosine triphosphate), which is utilised for growth, maintenance of tissues, aerobically fuelled exercise, and recovery from fatigue/maximum locomotor capacity (Pelletier et al., 1993). Therefore, decreases in V_{mt} may limit the amount of oxygen available to swimming muscles, and indeed, this change has also been associated with decreases in whole-animal metabolic rates (e.g., oxygen uptake rates; $\dot{M}O_2$), especially during exercise. Changes in swimming performance and oxygen uptake rates that are driven by changes in muscle V_{mt} may not pose as much of a challenge for pelagic larvae that metamorphose to less-active lifestyles as juveniles (e.g., flatfishes and cods) and do so to deeper benthic habitats with only mild changes in current velocity. However, for larval fishes settling onto shallow water habitats, such as coral reefs that experience fast current velocities and high, unpredictable wave-action, any rapid changes in swimming performance, $\dot{M}O_2$, and V_{mt} would pose a more significant challenge. These metrics together have not been measured in tropical coral reef fish larvae/early juveniles, despite their involvement in key challenges that characterise metamorphosis and recruitment processes among coral reef fishes that make them better suited for their new reef home.

Reef fishes are physiologically and morphologically well-suited for the hydrodynamic conditions on coral reefs. Recently, it has been suggested that adult reef fishes, such as the red shoulder wrasse (*Stethojulis bandanensis*), that inhabit shallow reefs with high unpredictable wave action, swim at high swimming speeds and have the highest factorial aerobic scope (FAS; the fold increase between rest and maximum $\dot{M}O_2$) relative to other pelagic fishes including tunas (Fulton et al., 2013). These fishes, like many other reef-associated species, achieve these high, prolonged speeds by employing labriform swimming, which is an energetically costly swimming mode that relies heavily on the pectoral fins for lift to combat intense currents (Fulton et al., 2013). In contrast, when pelagic settlement-stage larvae swim toward a reef, they primarily use body-caudal fin movements that incorporate body trunk musculature to achieve high steady speeds against oceanic currents (Hale et al., 2006). Body-caudal fin swimming, however, may not be as suitable to cope with intense wave action associated with the reef environment (Fulton et al., 2013). Although, labriform swimming may not be suitable for larval or newly settled juvenile fish, as it has been shown that pectoral muscles may not yet be fully developed in larval stages (Green et al., 2011; Patterson et al., 2008; Thorsen & Hale, 2005). Moreover, pectoral swimming can only be energetically sustained at speeds slower than average currents around wave-swept reefs (Hale et al., 2006). Therefore, the rapid decreases in performance associated with metamorphosis in reef fishes may represent a critical transition period, characterised by a shift in V_{mt} among specific swimming muscles. Body trunk V_{mt} may begin to decrease upon metamorphosis, and pectoral V_{mt} may begin to increase as newly settled larvae adjust to the new hydrodynamic regimes on reefs; however, this shift may impede

swimming while this transition occurs. Taken together, metamorphosis-associated changes in muscle V_{mt} may leave larvae vulnerable to predation after exhaustive exercise or unable to achieve speeds high enough to escape predators or combat reef waves and currents. These challenges may highlight the vulnerability reef fish larvae experience during metamorphosis and why this stage is a critical bottleneck for their survival.

Aims

In this study I investigated reef fish metamorphosis from a physiological perspective by measuring metrics of whole animal performance (swimming performance, oxygen uptake rates) and the cellular processes mediating performance (V_{mt} in pectoral and trunk musculature) in larval and settled juvenile Ambon damselfish (*Pomacentrus amboinensis*), a reef-associated coral reef fish species that primarily uses labriform swimming as an adult. These physiological metrics may predict how reef fishes transition their energy use when they transition from the pelagic environment to the benthic reef. These habitats are distinct in their hydrology, and therefore, fishes may require different oxygen demands to successfully develop and swim. Correlating oxygen uptake rates, specifically aerobic scope (described in **chapter 5**) and cost of transport (CoT; the amount of oxygen required for an animal to move a unit distance; Schmidt-Neilson 1971) with changes in muscle architecture (e.g., muscle mitochondria volume density) as reef fishes transition from the pelagic environment to the reef may provide the mechanistic link that supports metamorphosis. I measured these metrics in larvae just prior to settlement and juveniles that had settled for 24, 48, and 72 hours to determine: i) how swimming performance changes upon settlement, ii) whether energy expenditure ($\dot{M}O_2$) supporting swimming changes upon settlement, and iii) whether V_{mt} changes between trunk and pectoral musculature of these fishes occur as they transition to the reef. I follow hypothesis 4 of my thesis, and hypothesize that any measurable decreases in swimming performance upon settlement may be associated with shifts in mitochondrial volume densities between the trunk and pectoral muscle tissue types, which may limit oxygen availability to swimming muscles as the fishes adjust to transitioning from the open ocean to their new reef home. I predict that oxygen uptake rates decrease around the metamorphosis event, as living on the reef may be less energetically demanding when compared to swimming in the open ocean.

6.3 Methods

6.3.1. Fish collection and husbandry

Fish collection and experimentation occurred between November and December 2018 in the northern Great Barrier Reef at Lizard Island Research Station, which is located 240 km off the shore of Cairns, Australia (14°40’S, 145°28’E). Juvenile and larval Ambon damselfish (*Pomacentrus*

amboinensis) were collected from the fringing reef using light traps (8-16m depth). Traps were deployed in the afternoon at approximately 1600h and subsequently collected at night, around 2200h, to capture larvae and the following morning at approximately 0600h to capture juveniles. Pre-settlement larvae were identified using methods outlined by Murphy et al., (2007). Upon capture, all individuals were gently placed into 40L flow-through aquaria at the research station (28°C, natural photoperiod; 4 individuals L^{-1}); all aquaria were clearly labelled as to when they were captured and their corresponding post-settlement juvenile group (i.e., 24, 48, or 72h post-settlement). Juvenile Ambon damselfish were tested for swimming performance and oxygen uptake rates during swimming, several days post-settlement to determine the point at which swimming performance changes during the metamorphosis period when compared to pelagic larvae, and if a decrease in swimming performance corresponds with changes in oxygen uptake rates. Juveniles were fed *Artemia* spp. nauplii, twice daily, and aquaria were cleaned every three days. Food was withheld from individuals for 12 hours prior to experimentation to limit the impacts of specific dynamic action on respiration rates (McLeod et al., 2013). All ethics to capture, maintain, and experiment upon these animals were approved by James Cook University's Animal Ethics Committee (ethics approval number A2408).

6.3.2. Swimming respirometer

The individuals used in this experiment were swum in the same respirometer as in **chapter 5**. However, since there were differences in body size between studies, the specifications of the respirometer are described. Individual fish were swum using a glass, Blazka-style swimming respirometer (volume (V) =125ml; length (L) = 14.5cm; diameter (⌀) = 2.7cm), which provides a simultaneous estimate of oxygen uptake rates ($\dot{M}O_2$) while an individual swims at any given speed. The swimming respirometer was calibrated prior to experimentation, using a high-speed camera and passive particles. To reduce the volume of the respirometer, providing a more accurate $\dot{M}O_2$ measurement for the size of animals swum, an insert (⌀ = 2.6cm, L= 5.5 cm) was placed in the respirometer to create the working section. The working section of the respirometer (V = 50 ml, L= 6.5cm, ⌀ = 2.7cm) fit a smaller chamber, where an individual fish swum (volume with swimming chamber = 40 ml). This smaller chamber (V = 5.1ml, L = 4.5cm, ⌀ = 1.2cm) was fitted with a flow straightener (made from capillary tubes of ⌀ = 1.1mm, L = 40mm) to mitigate micro-turbulent flow, and a downstream mesh barrier was positioned to prevent the individual fish from being sucked into the propeller (mesh ⌀ = 0.415 mm). The swimming chamber was large enough for an individual fish to swim in any direction comfortably (mitigate stress of enclosure), and prevented blocking effects (<5%) which would alter the flow within the chamber. An external flush pump was used to deliver clean seawater (temperature (T) = 28°C; pressure (P) = ~1 bar; salinity (S) = 33ppt, dissolved oxygen (DO) = ~6.1 mg ml^{-1}) to the system in between measurement periods (see

Experimental protocol for details). Oxygen uptake (mg O_2 ml^{-1}) and T (°C) was simultaneously measured using respective probes (oxygen probe: OXROB3 Robust Oxygen Probe, PyroScience, Achen Germany; T sensor: TSUB36 Shielded submersible temperature sensor, Pyroscience, Achen, Germany). Oxygen probes were calibrated to 100% air saturation using fully aerated seawater (T = 28°C, S = 33ppt, P = 1bar, DO = 6.1mg O_2 ml^{-1}) and to 0% oxygen saturation (T = 28°C, S = 33ppt, P = 1 bar, DO = 0mg O_2 ml^{-1}) using sodium sulphite (Na_2SO_3; UNIVAR Analytical Reagent, Ajax Finechem, New South Wales, Australia). Oxygen and temperature probes were connected to a Firesting oxygen meter (4-channel optical oxygen meter, Pyroscience, Achen Germany), which constantly measured both of these variables throughout each experiment (a reading for temperature and oxygen was provided every second). An outer temperature jacket (V = 85ml, L = 6.5 cm) maintained water temperature at experimental conditions (T = 28°C), even at high water velocities. Prior to any experimentation, all components of the swimming respirometer were washed in a 10% bleach solution to kill any bacteria in the system.

6.3.3. Experimental protocol

The same experimental protocol was used for larval (n=10) and juvenile (24h, 48h, and 72h post-settlement; n=10 per settlement age) Ambon damselfish. However, larvae were swum during the night to prevent metamorphosis. The respirometer was blocked off from the rest of the experimental room using a curtain to prevent external stimuli from stressing the fish. Prior to each individual experiment, background respiration (i.e., oxygen uptake rates of bacteria) was measured for ten minutes (Rummer et al., 2016). To mitigate bacterial respiration, seawater (28°C) used for all experiments was UV-filtered (Blagdon Pro 24W ultra-violet clarifier, Dreative Pumps, South Australia, Australia). An individual fish was then placed in the swimming chamber and then quickly into the working section of the swimming respirometer. The entire swimming respirometer was sealed underwater to prevent air bubbles from building up in the system. Each individual was allowed to habituate to the chamber and rest from handling/transport stress for 30 min, at a water velocity equivalent to 1 body length per second (BL sec^{-1}; a subsample of individuals (n=10 per settlement stage) was measured (total length; snout to tip of caudal fin) to provide an overall estimate for body length) (Brett, 1964). A black tarp was draped over the chamber to further mitigate stress during the habituation period. Individuals underwent a stepped velocity test, post-habituation, to measure critical swimming speed (U_{crit}), a test designed to estimate the aerobic capacity of fishes (Brett, 1964). Every 20 min, the water velocity in the chamber increased by 1 BL sec^{-1} until the individual fish fatigued, as indicated by impingement on the downstream barrier. Critical swimming speed was calculated using the following formula:

$$U_{crit}\ (\text{cm sec}^{-1}) = Vf + (T/t)*Vi$$

Where, Vf is the penultimate speed (cm sec^{-1}), T is the time swum at the fatigue speed, t is the time interval (20 minutes) and Vi is the velocity increment (approximately 1.5 cm sec^{-1}) (Brett, 1964). At each swimming speed, $\dot{M}O_2$ was measured using intermittent flow respirometry, consisting of a 20 minute measurement period (time interval portion of the U_{crit} protocol), followed by a 3 minute flush period (replenish swimming chamber with clean, aerated seawater; occurs once water velocity increases to next speed) (Rummer et al., 2016). The flush period was long enough for oxygen within the swimming chamber to be replenished to 100% air saturation (DO=6.1mg O_2 ml^{-1}). Oxygen within the swimming chamber was never allowed to fall below 90% air saturation to prevent oxygen uptake rates of the fish being influenced by hypoxia (Rummer et al., 2016). Upon completion of their respective U_{crit} protocol, each individual fish was removed from its respirometer, euthanized in an ice bath (see below for details), and then a subsequent reading for any respiration by accumulated bacteria in the chamber introduced by the fish was performed for 10 minutes (Rummer et al., 2016).

Text files from the Firesting were imported and analysed in LabChart (ver 8, ADinstruments, New South Wales, Australia) to calculate $\dot{M}O_2$ at each swimming speed. The oxygen uptake rate ($\dot{M}O_2$) at each 20-minute interval was calculated as:

$$\dot{M}O_2\ (\text{mg } O_2\ \text{kg}^{-1}\ \text{h}^{-1}) = S \bullet V_{resp} M^{-1}$$

where S is slope of the linear regression during the measurement period (mg O_2 s^{-1}), V_{resp} is the volume of the respirometer (minus the fish), and M is the mass of the individual fish (kg) (Rummer et al., 2016). $\dot{M}O_2$ was expressed as mg O_2 kg^{-1} h^{-1}, and each $\dot{M}O_2$ value was plotted against swimming speed (cm sec^{-1}). The appropriate linear regression (1) or power curve (2) was fit through this relationship:

$$(1)\ R(u) = a + bu$$

$$(2)\ R(u) = a + cu^b$$

Where R(u) is an estimate of mass-specific oxygen uptake (mg O_2 $kg^{-1}h^{-1}$) at any given speed (u; cm sec^{-1}), a is the y-intercept, b is the slope of the equation, and c is an estimated parameter (Brett, 1964; Hunt von Herbing et al., 2001; Rummer et al., 2016). The y-intercept of this relationship between oxygen uptake and swimming speed provides an estimate of the fish's standard metabolic rate (SMR; mg O_2 kg^{-1} h^{-1}; Chabot et al., 2016), which is an estimate of the basic metabolic functions of the animal at rest (u=0 cm sec^{-1}). The maximum $\dot{M}O_2$ value when the individual fish fatigued, is an estimate of maximum metabolic rate (MMR; mg O_2 $kg^{-1}h^{-1}$; Norin & Clark, 2016), which is also an estimate of the fish's maximum sustainable (i.e., aerobic) swimming speed. Aerobic scope (AS; mg O_2 $kg^{-1}h\text{-}^{1}$) was calculated

by subtracting SMR from MMR, and is an estimate of the capacity of the fish to perform all relevant aerobically driven tasks (Brett, 1964; Rummer et al., 2016). Cost of transport provides an estimate of the amount of energy the fish needs move a unit distance (Schmidt-Nielsen, 2016), and here, COT at each velocity interval during the U_{crit} test was calculated as the following:

$$\text{COT (mg } O_2 \text{ kg}^{-1} \text{ cm}^{-1}) = \dot{M}O_2/U$$

where $\dot{M}O_2$ is oxygen uptake rate (mg O_2 kg^{-1} s^{-1}), and U is swimming speed (cm s^{-1}) (Vagner et al., 2008).

6.3.4. Morphometrics tissue collection

Immediately after euthanasia, fish were measured for total length (tip of snout to tip of caudal fin), blotted dry and weighed (g) using a scale (Kern ABJ-NM/ABS-N, Kern, Balingen, Germany). Fish were then subsequently placed in a 1.5ml vial containing glutaraldehyde in a phosphate buffer (2% glutaraldehyde and 2% paraformaldehyde, phosphate buffer pH =7.4, ProSciTech, Townsville, Australia) for tissue fixation (Hernández et al., 2016). After 24 hours, a thin layer of red muscle from the left and right sides of the trunk musculature of the fish was dissected under a dissecting microscope. Pectoral fin adductor muscle groups were also removed from both pectoral fins of the fish (Hernández et al., 2016). Muscle tissue samples were then placed in a fresh vial of glutaraldehyde and phosphate buffer for tissue fixation.

6.3.5. Quantifying mitochondria volume density

Electron microscopy (Transmission Electron Microscopy; TEM) was performed at the Advanced Analytical Center at James Cook University (Cairns, Queensland, Australia). After fixation, muscle tissue samples were rinsed several times in fresh phosphate buffer with no fixative before being post-fixed for one hour in 1% aqueous osmium tetroxide solution. Specimens were then rinsed with deionized (DI) water before being *en bloc* stained with ethanoic 5% uranyl acetate for 1-2 hours at room temperature in the dark. Specimens were then rinsed with DI water prior to dehydration with a graded ethanol series (50, 70, 80, 90, and 100%). At the 100% stage, the ethanol solution was changed three times and left for 15 min at each time. The specimens were then transferred to a 2:1 solution of 100% ethanol and Epon resin for 15 min, followed by a 1:1 solution of 100% ethanol and resin, and then by a 2:1 solution of Epon and 100% ethanol. The specimen was then submerged in three fresh changes of 100% resin for 15 min each before being left on a rotator in fresh 100% resin overnight. The following morning the samples were embedded into silicon molds with fresh resin and polymerized in an oven at 60°C for 24 hours. Resin blocks were then cut on an ultramicrotome (Leica UC7 Ultramicrotome, Leica biosystems, Nussloch,

Germany). Thick sections (approximately 1 μm) were first cut with glass knives, placed on slides, and stained with toluidine blue to assess orientation and presence/absence of areas of interest. Thin sections (50-70 nm) were then cut with a diamond knife and transferred to copper TEM grids. Sections on grids were then post stained with uranyl acetate and lead citrate to increase contrast before viewing (1500X zoom) on a transmission electron microscope (Hitachi 7800 Transmission electron microscope, Hitachi High-Tech Global) and photographed. For each sample of tissue, 10 micrographs were taken.

Micrographs of mitochondria were uploaded into ImageJ. A grid was superimposed onto each micrograph. Grid calibration was performed to determine the proper grid line spacing size to count mitochondria, by placing grids with increasing sized spaces between lines. I selected the largest grid size (ImageJ grid size setting = 0.02; total number of intersecting points on grid = 3888) that yielded the same number of mitochondria as grid spacing size decreased. I used a point count method to quantify mitochondria for each micrograph (Weibel, 1980). For each micrograph I first started by counting any points that were outside of the cell membrane, and subtracted that value from the total number of points (3888) to get the total number of points on the micrograph (P_{total}). I then counted the number of intersecting points that landed on mitochondria in the micrograph (P_{mt}). Muscle mitochondria volume (V_{mt}) was calculated using the following formula (Weibel, 1980):

$$V_{mt} = (P_{mt}/P_{total})*100$$

The V_{mt} from each of the 10 micrographs were average together to calculate a representative mean V_{mt} for each tissue (Weibel 1980).

6.3.6. Statistical Analysis

All data were first checked for normality using Shapiro-Wilk test. Critical swimming speed (U_{crit}), $\dot{M}O_2$ (SMR, MMR, AS) and V_{mt} were analyzed using a one-way analysis of variance (ANOVA), with stage (larvae, 24, 48, 72h juveniles) as a fixed factor. Cost of transport (COT) was analyzed using a two-way ANOVA, investigating the interaction of swimming speed and COT among stage. Mitochondria volume density (V_{mt}) from trunk and pectoral musculature were analyzed using two-way ANOVA, investigating the interaction of muscle type on V_{mt} among stage. Swimming speed and stage were fixed factors for COT analyses. Muscle type (i.e., trunk or pectoral) and stage were fixed factors for V_{mt} analyses. All statistical analyses were run in R (ver 3.6.1. http://www.R-project.org/), at $\alpha = 0.05$.

6.4 Results

No significant differences in U_{crit} (mean ± s.e.) were detected between between larvae (24.9± 0.7 cm s^{-1}) and juveniles settled for 24 h (24.3±1.1 cm s^{-1}) or 48 h (24.6±1.2 cm s^{-1}) ($p > 0.8$; Figure 6.1, Table

6.1, 6.2). However, 72h post-settlement fish exhibited a 50% decrease in U_{crit} when compared to larvae and 24 and 28h post-setttlement juveniles ($p<0.001$; Figure 6.1, Table 6.2).

Standard Metabolic Rate (SMR), decreased in fish upon settlement, but this trend was not significant ($p>0.3$ for all combinations of stages; Figure 6.2a, Table 6.3, 6.4). The SMR (mean ± s.e.) across developmental stages ranged as follows: larvae, 1508.4±174.6 mg O_2 kg^{-1} h^{-1}; 24 h juvenile, 1007.5±120.7 mg O_2 kg^{-1} h^{-1}; 48 h juvenile, 1092.1±88.3 mg O_2 kg^{-1} h^{-1}; 72 h juvenile, 1104.5±151.1 mg O_2 kg^{-1} h^{-1} (Figure 6.2a). A similar non-significant decreasing trend was observed in estimates of maximum metabolic rate (MMR) ($p>0.1$ for all combinations of stages; Figure 6.2b, Table 6.5, 6.6). The MMR (mean ± s.e.) across developmental stages ranged as follows: larvae, 3111.2±200 mg O_2 kg^{-1} h^{-1}; 24 h juvenile, 2429.2±164.9 mg O_2 kg^{-1} h^{-1}; 48 h juvenile, 2328.1±166.6 mg O_2 kg^{-1} h^{-1}; 72 h juvenile, 2354.4±181.5 mg O_2 kg^{-1} h^{-1} (Figure 6.2b). Absolute aerobic scope (AAS), was maintained across all stages ($p>0.2$ across all combinations of stages; Figure 6.2c, Table 6.7, 6.8). The AS (mean ± s.e.) across developmental stages ranged as follows: larvae, 1602.8±142.2 mg O_2 kg^{-1} h^{-1}; 24 h juvenile, 1421.7±136.8 mg O_2 kg^{-1} h^{-1}; 48 h juvenile, 1236±189.9 mg O_2 kg^{-1} h^{-1}; 72 h juvenile, 1249.9±170.7 mg O_2 kg^{-1} h^{-1} (Figure 6.2c).

There were no differences in COT between larvae and 72-hour juveniles ($p=0.8$), or between 24 and 48 h juveniles ($p=0.95$) (Figure 6.3, Table 6.9, 6.10). However, 24 and 48 h juveniles showed significantly lower COT than larvae ($p<0.001$) and 72-hour juveniles ($p>0.001$) (Figure 6.3, Table 6.10). As swimming speeds increased, COT decreased significantly ($p>0.001$ across all stages, Table 6.9). An asymptote was observed, prior to fatigue, for all stages (Figure 6.3).

Mitochondrial volume density (expressed as a %, V_{mt}), is significantly influenced by muscle type ($p<0.005$) and settlement stage ($p<0.005$; Figure 6.4 Table 6.11). Muscle V_{mt} was higher in pectoral muscles than in trunk musculature at all observed settlement stages (Figure 6.4, Table 6.12). Larvae prior to metamorphosis, have pectoral V_{mt} of 9.1±1.3% (mean ± s.e.) and trunk V_{mt} of 4.2±0.1% mean ± s.e. (Figure 6.4). There is a significant increase in both pectoral and trunk V_{mt} within 24 h of settlement (pectoral: average V_{mt} = 23.3±2.9%, $p=0.003$; trunk: average V_{mt} = 9.7±1.5%, $p=0.002$) (Figure 6.4, Table 6.12). Within 48 h, there was a decrease in V_{mt}, in both pectoral and trunk muscle; however, this decrease was not significantly different than V_{mt} in 24 h juveniles (trunk: average V_{mt} = 6.1±0.4%, $p= 0.27$; pectoral: average V_{mt} = 17.4±3.1%, $p=0.36$) (Figure 6.4, Table 6.12). By 72 h, V_{mt} in both tissues decreased back down to densities similar to pelagic larvae, which were significantly lower than 24 h settled juveniles (trunk: average V_{mt} = 3.4±1.1%, $p=<0.005$; pectoral: average V_{mt} = 12.5±5.9%, $p= 0.03$) (Figure 6.4, table 6.12).

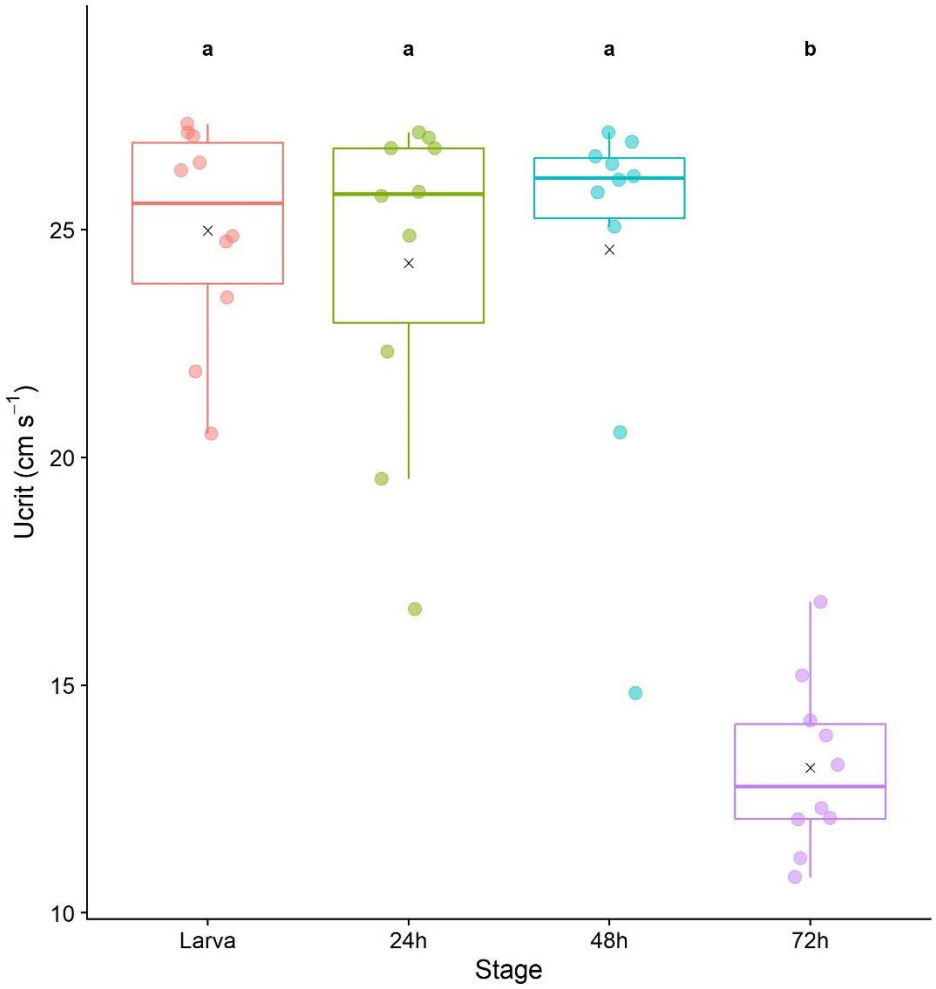

Figure 6.1 Critical swimming speed (U_{crit}; cm sec^{-1}) of larval (n=10) and juvenile Ambon damselfish (*Pomacentrus amboinensis*). Juvenile fishes were swum 24, 48, and 72 h post-settlement (n=10 per settlement stage). Boxplots show median and interquartile ranges. Each point represents one measurement per individual. Mean values are represented by an 'x' within each bar, and different letters represent statistical differences (α=0.05).

Table 6.1 Summary statistics for the differences in critical swimming speed (U_{crit}) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*)

Model=(Ucrit~Stage,data)

	Estimate	**Std. error**	**t-value**	**p**
Intercept	24.3	0.97	25.1	<0.0001
stage48h	0.29	1.4	0.22	0.83
stage72h	-11.1	1.4	-8.1	<0.0001
stageLarva	0.71	1.4	0.52	0.61

$F_{3,36}$=34.9

Table 6.2 Pairwise comparison, using Tukey's post-hoc test, of the differences in critical swimming speed (U_{crit}) between four settlement stages(larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*).

Comparison	**Estimate**	**SE**	**Df**	**t.ratio**	**p**
24h – 48h	-0.294	1.37	36	-0.215	0.994
24h – 72h	11.1	1.37	36	8.1	<0.0001
24h – Larva	-0.71	1.37	36	-0.518	0.951
48h – 72h	11.4	1.37	36	8.32	<0.001
48h – Larva	-0.415	1.37	36	-0.303	0.99
72h – Larva	-11.8	1.37	36	-8.62	<0.001

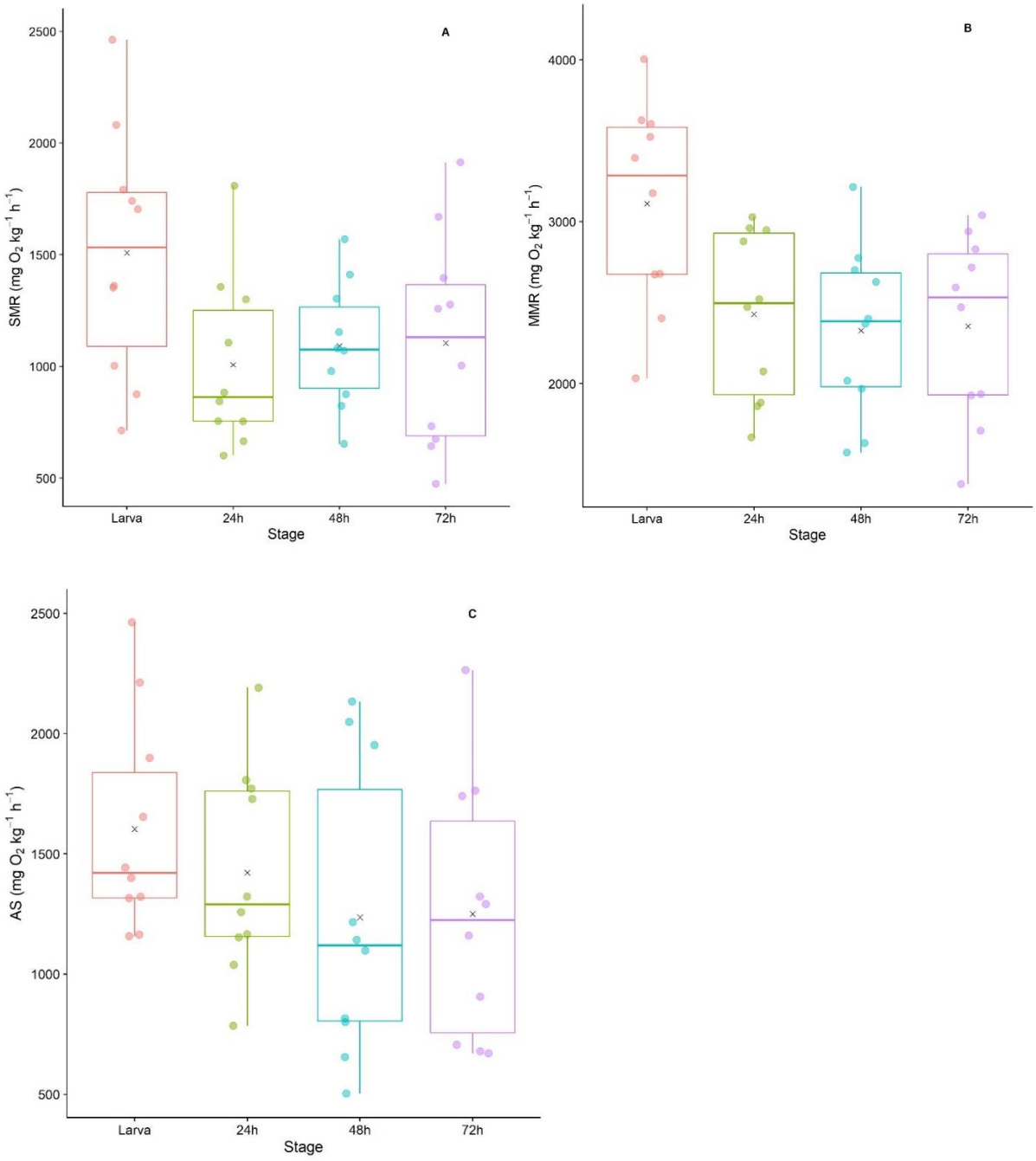

Figure 6.2 Estimates of A) standard metabolic rate (SMR; mg O_2 kg^{-1} h^{-1}) and b) maximum metabolic rate (MMR; mg O_2 kg^{-1} h^{-1}) and calculated c) aerobic scope (AS; mg O_2 kg^{-1} $h^{-}1$) of larval (n=10) and juvenile Ambon Damselfish (*Pomacentrus amboinensis*). Juvenile fishes were swum 24, 48, and 72 h post-settlement (n=10 individuals per settlement stage). Each point per stage represents one measurement per individual. Boxplots represent median and quartile ranges. Mean values are represented by an 'x' within each bar.

Table 6.3 Summary statistics for the differences in standard metabolic rate (SMR) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*)

Model=(SMR~Stage,data)

	Estimate	**Std. error**	**t-value**	**p**
(intercept)	999.7	449	2.2	0.033
Stage48h	87.3	224.2	0.39	0.69
Stage72h	103.2	214.9	0.48	0.63
stageLarva	508.5	256.9	1.9	0.056

$F_{5,34}$ =1.516

Table 6.4 Pairwise comparison, using Tukey's post-hoc test, of the differences in standard metabolic rate (SMR) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*).

Comparison	**Estimate**	**SE**	**Df**	**t.ratio**	**P**
24h – 48h	-87.3	224	34	-0.39	0.98
24h – 72h	-103.2	215	34	-0.48	0.963
24h – larva	-509	257	34	-1.98	0.216
48h – 72h	-15.9	203	34	-0.078	0.999
48h – larva	-421	209	34	-2.01	0.204
72h - larva	-405	217	34	-1.87	0.262

Table 6.5 Summary statistics for the differences in maximum metabolic rate (MMR) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*)

Model=(MMR~Stage,data)

	Estimate	**Std. error**	**t-value**	**p**
(intercept)	7.8	0.075	104.1	<0.0001
Stage48h	-0.044	0.11	-0.42	0.67
Stage72h	-0.039	0.11	-0.37	0.71
StageLarva	0.25	0.11	2.4	0.024

$F_{3,36}$=3.5

Table 6.6 Pairwise comparison, using Tukey's post-hoc test, of the differences in maximum metabolic rate (MMR) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*).

comparison	**Estimate**	**SE**	**Df**	**t.ratio**	**P**
24h – 48h	0.0446	0.106	36	0.422	0.974
24h – 72h	0.0393	0.106	36	0.372	0.982
24h – larva	-0.247	0.106	36	-2.36	0.103
48h – 72h	-0.00529	0.106	36	-0.05	1.0
48h – larva	-0.293	0.106	36	-2.78	0.0506
72h - larva	-0.293	0.106	36	-2.73	0.0556

Table 6.7 Summary statistics for the differences in absolute aerobic scope (AAS) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*)

Model=(AS~Stage,data)

	Estimate	**Std. error**	**t.value**	**p**
(intercept)	2010.3	511.5	3.9	<0.0001
Stage48h	-27.6	255.4	-0.11	0.91
Stage72h	-41.9	244.9	-0.17	0.86
StageLarva	440.7	292.6	1.5	0.14

$F_{5,34}$=1.1

Table 6.8 Pairwise comparison, using Tukey's post-hoc test, of the differences in absolute aerobic scope (AAS) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*).

Comparison	**Estimate**	**SE**	**Df**	**t.ratio**	**p**
24h – 48h	27.6	255	34	0.108	0.999
24h – 72h	41.9	245	34	0.171	0.998
24h – larva	-441	293	34	-1.51	0.445
48h – 72h	14.3	231	34	0.062	0.999
48h – larva	-468	238	34	-1.96	0.221
72h - larva	-483	248	34	-1.95	0.227

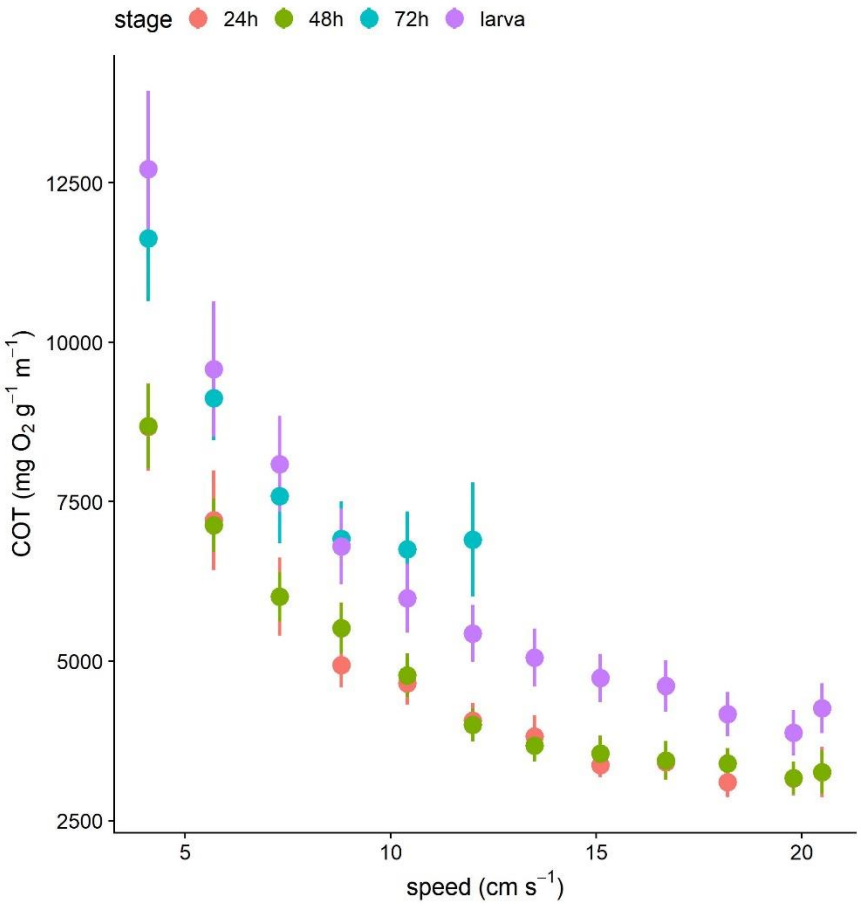

Figure 6.3 Cost of transport (COT; mg O_2 $g^{-1}m^{-1}$) of larval (n=10 individuals) and juvenile Ambon damselfish (*Pomacentrus amboinensis*) swimming at incremental swimming speeds (cm s^{-1}) during a critical swimming test (U_{crit}). Juveniles were swum 24, 48, and 72 h post settlement (n=10 individuals per stage). The last point on each curve represents the maximum swimming speed before fatigue.

Table 6.9 Summary statistics for the differences in Cost of Transport (COT) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*) experienced at increasing speeds during a critical swimming test.

Model=lm(COT~Stage+speed)

	Estimate	**Std.error**	**t-value**	**p**
(intercept)	9243.7	304.8	30.3	<0.0001
Speed	-374.7	20.7	-18.1	<0.0001
Stage48h	134.5	263.3	0.51	0.61
Stage72h	2048.4	349.6	5.9	<0.0001
StageLarva	1735.3	258.6	6.7	<0.0001

$F_{4, 359} = 124.7$

Table 6.10 Pairwise comparison, using Tukey's post-hoc test, of the differences in Cost of Transport (COT) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*).

Comparison	**Estimate**	**SE**	**Df**	**t.ratio**	**P**
24h - 48h	-135	263	359	-0.511	0.957
24h – 72h	-2048	350	359	-5.86	<0.0001
24h – larva	-1735	259	359	-6.71	<0.0001
48h – 72h	-1914	351	359	-5.45	<0.0001
48h – larva	-1601	259	359	-6.18	<0.0001
72h – larva	313	349	359	0.898	0.806

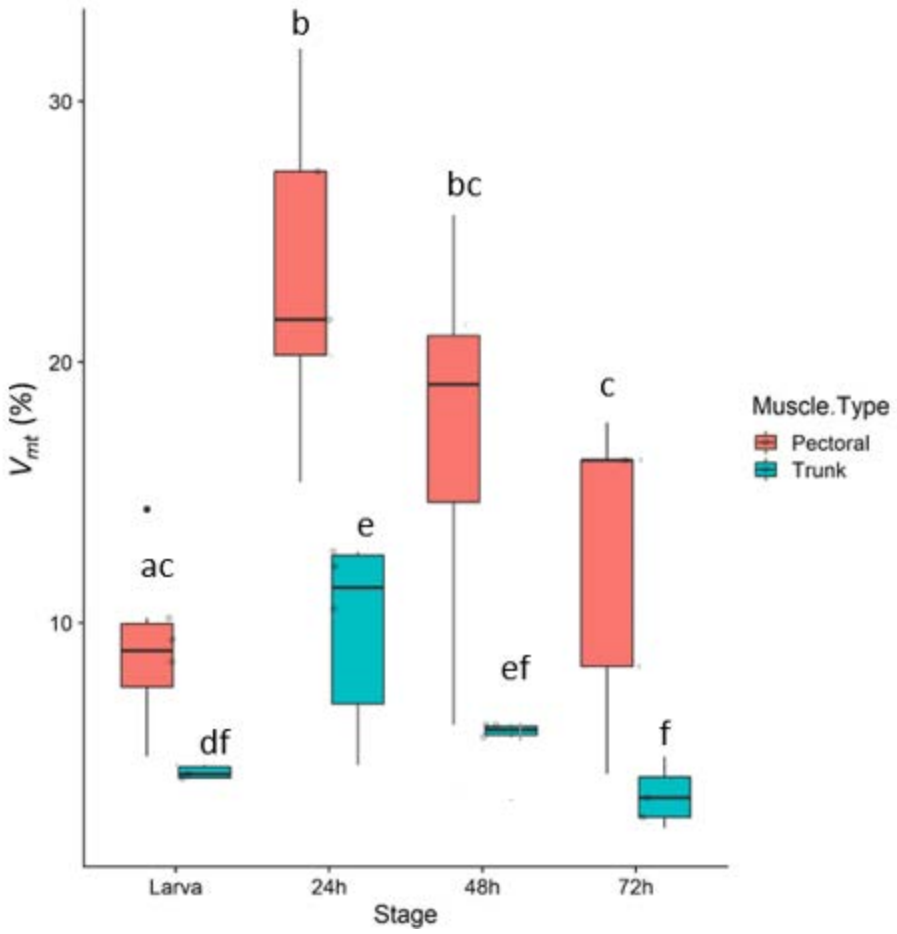

Figure 6.4 Mitochondrial volume density (V_{mt}) taken from pectoral (red) and trunk (blue) muscles from larval (n=10) and juvenile (24, 48, and 72 h settlement stage; n=10 individuals per stage) Ambon damselfishes (*Pomacentrus amboinensis*). Boxplots represent median and interquartile ranges. Different letters represent statistical differences (α=0.05).

Table 6.11 Summary statistics for the differences in muscle mitochondrial volume density (V_{mt}) of two tissue types (pectoral and trunk muscle) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*).

Model=(Vmt~Stage+muscle.typ)

	Estimate	**Std.error**	**t.value**	**p**
(intercept)	21.3	1.6	13.7	<0.00001
Muscle.Type	-9.9	1.4	-7.3	<0.00001
Stage48h	-4.8	1.9	-2.6	0.014
Stage72h	-8.4	1.9	-4.3	0.00013
StageLarva	-9.9	1.9	-5.2	<0.00001

$F_{4,39} = 19.9$

Table 6.12 Pairwise comparison, using Tukey's post-hoc test, of the differences in muscle mitochondria volume density (V_{mt}) in two muscle types (pectoral and trunk) between four settlement stages (larva, 24h, 48h and 72h juveniles) for ambon damselfish (*Pomacentrus amboinensis*).

Comparison	**Estimate**	**SE**	**Df**	**t.ratio**	**p**
LarvaPec – LarvaTrunk	9.92	1.36	39	7.27	<0.0001
24hPec – 24hTrunk	9.92	1.36	39	7.27	<0.0001
48hPec – 48hTrunk	9.92	1.36	39	7.27	<0.0001
72hPec – 72hTrunk	9.92	1.36	39	7.27	<0.0001
24hPec – 48hPec	4.82	1.89	39	2.56	0.0668
24hPec – 72hPec	8.41	1.97	39	4.26	0.0007
24hPec – larvaPec	9.93	1.93	39	5.15	<0.0001
48hPec – 72hPec	3.59	1.93	39	1.86	0.236
48hPec – larvaPec	5.11	1.89	39	2.71	0.0471
72hPec - larvaPec	1.52	1.97	39	0.77	0.867
24hTrunk – 48hTrunk	4.82	1.89	39	2.56	0.06
24hTrunk – 72hTrunk	8.41	1.97	39	4.26	0.0008
24hTrunk – larvaTrunk	9.93	1.93	39	5.15	<0.0001
48hTrunk – 72hTrunk	3.59	1.93	39	1.86	0.4
48hTrunk – larvaTrunk	5.11	1.89	39	2.71	0.002
72hTrunk - larvaTrunk	1.52	1.97	39	0.77	0.9

6.5 Discussion

This chapter aimed to investigate some of the physiological mechanisms underpinning metamorphosis of reef fishes, and addressed hypothesis 4, which stated that reef fishes would lower their oxygen uptake rates and lower muscle mitochondria volume densities to nel to a benthic existence. The pelagic to reef transition period remains one of the least understood aspects of coral reef fish ecology (Leis et al., 2013a), but is important because this period is a critical bottleneck for reef fish population dynamics due to high predation-related mortality. Therefore, reef fish larvae need to rapidly metamorphose to be best suited for the challenges associated with their reef environment (McCormick & Makey, 1997; McCormick et al., 2002). While the physiology underpinning metamorphosis has not been heavily investigated, it is now understood that metamorphosis encompasses more than just morphological changes (e.g., loss of spines or colour change). In fact, as demonstrated in this chapter, coral reef fishes quickly alter their physiology upon settlement, although quite different to my hypothesis. Oxygen uptake rates did not decrease upon settlement and Cost of Transport decreased temporarily in response to an increase in muscle mitochondria volume densities. While the decreases in swimming performance observed here support previous findings (e.g., Leis et al., 2011), the current study is the first to investigate the way in which oxygen uptake rates supporting swimming and muscle mitochondria change as pelagic reef fish larvae settle onto a reef and metamorphose into juveniles. These rapid changes in their physiology may provide a more mechanistic understanding as to how reef fishes successfully make the successful transition from the open ocean to benthic reef habitats.

6.5.1 Changes in swimming performance associated with metamorphosis

Ontogenetic changes in swimming performance during the larval to juvenile transition and upon metamorphosis generally follow a pattern that depends on whether larvae exist in the same habitat as juveniles/adults. For pelagic fishes, swimming performance generally increases linearly with body size (e.g., body length) as larvae metamorphose into juvenile fishes. There are no latitudinal variations in this trend, as temperate pelagic fishes, such as Atlantic cod (*Gadus morhua*; Peck et al., 2006; Guan et al., 2008) and Atlantic herring (*Clupea harrengus*; Moyano et al., 2016), warm temperate pelagic fishes, such as gilthead seabream (*Sparus aurata*; Koumoundouros et al., 2009) and sardine (*Sardina pilchurus*; Silva et al*.,* 2014), and tropical pelagic fishes, such as mackerels (*Scomber japonicus* and *Trachurus japonicus*; Masuda, 2006), and giant trevally (*Caranx ignobillis*; Leis et al., 2006), all increase swimming performance with body size after metamorphosis. This trend has also been shown in demersal, non-reef fishes (Clark et al., 2005; Leis et al., 2007; Leis et al., 2009) as well as freshwater riverine species (Clark et al., 2005; Poulsen et al., 2012; Kopf et al., 2014; George et al., 2018). While the majority of these studies have measured swimming under laboratory conditions (e.g., routine swimming and/or U_{crit}), *in*

situ swimming performance observations in the field have been made for larval and juvenile giant trevally, and findings retain the aforementioned trends (Leis et al., 2006). Many studies have noted biphasic increases in swimming across ontogeny for fishes where swimming generally rapidly increases (i.e., exponentially or linearly) during the larval phase (1st phase) and then increases again, but less rapidly, after metamorphosis (2nd phase) (Peck et al., 2006; Moyano et al., 2016). This pattern greatly contrasts the ontogeny of swimming performance for many reef fishes.

Tropical reef fishes and some demersal fishes have been observed to undergo marked decreases in swimming performance immediately following metamorphosis of a pelagic larva into a benthic juvenile. In tropical reef fishes, this pattern was first observed in several pomacentrid species, whereby settled juveniles exhibited 31% slower critical swimming speeds than settlement stage larval conspecifics (Stobutzki & Bellwood, 1994). In two wrasse species, (*Thallassoma quinqueittatum* and *T. bifasciatum),* when pelagic larvae settled onto reefs as juveniles, within 24 h, they decrease median U_{crit} by 73% and by 83% by 48 h (Leis et al., 2011). Here, Ambon damselfish juveniles, 72 h post-settlement, swam, on average, 30-40% slower than larvae and 24 h or 48 h post-settlement juveniles. A rapid decrease in swimming performance under laboratory conditions (e.g., using U_{crit} or routine swimming) has been observed in several other reef and non-reef demersal species, from both temperate and tropical regions, such as European plaice (*Pleuronectes platessa*; Silva et al., 2015), shorthorn sculpin (*Myxocephalus scorpius*; Guan et al*.,* 2008), clingfishes (*Lepadogaster lepadogaster* and *L. porea*; Faria & Goṇalves, 2010), and blue-lined goatfish (*Upeneichthys lineatus*; Dudley et al., 2000). Additionally, Leis et al., (2013a) found that *in situ* swimming speed for orbicular batfish (*Platax orbicularis*) decreased as larvae approached settlement stage onto a reef. Interestingly, there are some exceptions, such as rockfishes (*Sebastes* spp.), which are temperate reef fishes that metamorphose into the juvenile phase in the water column and settle at a later stage, but increase swimming performance with size (Kashef et al., 2014). In the current study, the delayed decrease in swimming performance in fish at 72 h post-settlement highlights potential species-specific responses to settling onto a reef. Taken together, metamorphosis-induced changes in performance that occur as pelagic larvae move to a benthic lifestyle as a juvenile highlight that different physiological and behavioural modifications must be rapidly made to successfully transition to a new habitat.

Notable decreases in performance associated with demersal metamorphosis clearly emphasises the challenges associated with shifting from a pelagic to benthic habitat relative to simply remaining in the pelagic environment. Pelagic fishes increase swimming performance with growth, which is hypothesized as a strategy to remain in the pelagic and locate food patches, which are scarce (Llopiz, 2014; Silva et al., 2014). Similarly, the larvae of freshwater riverine species must swim against high flow

to locate specific nursery habitats (George et al., 2018). Several hypotheses have been put forward as to why metamorphosis induces decreases in performance in reef and non-reef demersal fishes. Metamorphosis is highly energetically expensive for flatfishes (e.g., eye migration), and reducing swimming may be a strategy to conserve energy reserves (Silva et al., 2015). Some temperate species change morphology (e.g., increase body depth and laterally compress the body) so they are more maneuverable in a complex benthic habitat (Koumoundouros et al., 2009; Leis et al., 2011). For coastal reef fishes, decreasing swimming performance has been hypothesized to be associated with tolerating nocturnal hypoxia events, and fast swimming performance may not be required to maneuver around corals (Nilsson et al., 2007a; Leis et al., 2011;). Rockfishes, which are a reef fish that metamorphoses in the water column as a strategy to improve dispersal potential (Kashef et al., 2014), are a notable exception. Ambon damselfishes in the current study face similar challenges as other reef fishes and also decrease their swimming performance upon settlement. However, it has been suggested that a decrease in performance is simply a behavioural response to metamorphosis, rather than a loss in whole-organism swimming ability (Faria & Goņalves, 2010; Silva et al., 2015). This hypothesis may be fully supported if oxygen uptake rates were made during swimming, which is definitely an avenue for future investigations.

6.5.2.1. Energy utilization across taxa of animals that undergo metamorphosis

Neither standard metabolic rate (SMR; energy associated with basic maintenance costs), maximum metabolic rate (MMR; highest oxygen uptake rate during exhaustive exercise), or aerobic scope (energy budget for all aerobically driven tasks) changed for Ambon damselfish prior to metamorphosis and 72 h post metamorphosis. This suggests that energy expenditure is tightly regulated during metamorphosis, and/or energy is allocated to other physiological tasks (e.g., growth, as described below). Such regulation of $\dot{M}O_2$ may be behaviourally mediated (see below). Energy use during metamorphosis is variable across diverse taxa of invertebrates and vertebrates. In the classic example of the Indian bullfrog (*Rana tigria*), oxygen uptake rates are high during the tadpole phase and then decrease during metamorphosis, as newly metamorphosed frogs rely additionally on endogenous energy reserves accumulated during the tadpole stage for energy versus oxygen uptake alone (Pandian & Marian, 1985). Similarly, $\dot{M}O_2$ decreased in metamorphosing larval ascidians (*Diplosoma listerianum*) as they switched to endogenous reserves for energy (Bennett & Marshall, 2005). Some invertebrates, such as abalone (Shilling et al., 1996) and oysters (Rodriguez et al., 1990), increase respiration rates during metamorphosis. In general, for many benthic animals with pelagic larvae, the cost of swimming (up to 25% of energy budget; Bennett & Marshall 2005) becomes too high to balance all physiological tasks, especially around onset of metamorphosis. Therefore, a decrease in respiration rate during metamorphosis is likely associated with

energy saving behaviours (Bryan, 2004). Oxygen uptake rates in marine fishes show a range of responses to metamorphosis.

Similar to invertebrates, oxygen uptake rates in marine fishes change in different ways during metamorphosis, which can depend particularly on the degree of morphological and anatomical changes the fish undergoes and the habitat on which the fish settles. For pelagic fishes, such as in the yellowtail kingfish (*Seriola lalandi*), absolute $\dot{M}O_2$ (i.e., not adjusting for mass) increases linearly across metamorphosis, (Moran, 2007). A similar trend has also been observed in demersal fishes, such as gilt-head sea bream (*Sparus auratua*; Parra & Yúfera, 2001). Studies on eels and red sea bream (*Pagrus major*) have noted decreases in oxygen uptake rates after metamorphosis, as the mass of metabolically active tissues (e.g., brain, viscera) decreases in proportion to whole body mass during periods of post-metamorphosis growth (e.g., much of a fish's body mass is white muscle, which is not as metabolically active) (Bishop & Torres, 1999; Ishibashi et al., 2005)). In contrast, lampreys increase $\dot{M}O_2$ during onset of metamorphosis, as they become active swimmers as juveniles after being buried under sediment as larvae (Lewis & Potter, 1977). Much of what we know about changes in respiration rates associated with metamorphosis, is from flatfishes (Pleuronectiformes), as they undergo dramatic changes in anatomy and behaviour between pelagic larval and juvenile stages. As flatfishes metamorphose, $\dot{M}O_2$ decreases, as energy is directed to morphological modifications (e.g., eye migration, gill formation), and less energy is dedicated to swimming as they transition to a benthic lifestyle (Cunha et al., 2007; Laurence, 1975). At this point in the discussion, however, it is also important to highlight methodological differences between studies. Most studies focusing on the physiological changes characterising metamorphosis investigate routine metabolic rate (RMR), which is estimated from oxygen uptake rates derived from undisturbed individuals. The current study estimated SMR, MMR and aerobic scope from critical swimming speed tests, as a proxy for energy use. Given that neither SMR, MMR, nor aerobic scope changed in this study suggests that: i) basic maintenance costs remain the same across metamorphosis, ii) oxygen uptake rates during fatigue caused by exhaustive exercise remain constant, and iii) energy budgets are not drastically altered by metamorphosis. Routine metabolic rates may be different based on activity levels upon settlement (described below), and it would be interesting to measure oxygen uptake rates under ecologically relevant flow speeds (Downie et al., 2021b). The decrease in U_{crit} after 72 h may suggest that energy is directed away from high performance swimming and toward other physiologically important tasks, such as growth, for successfully recruiting to adult populations on a reef.

Animals must be able to physiologically multitask (i.e., distribute appropriate amounts of energy) among many diverse functions (e.g., growth, performance, etc.) and systems (e.g., respiration, digestion, reproduction, etc.) to survive. Ontogenetic (e.g., life-stage specific) compromises in how energy is

distributed exist, and the temporary impairment of some functions allows others to be more effective. Larval teleost fishes are characterised by fast growth, while having limited aerobic scope (Killen et al., 2007; Cuhna et al., 2007), suggesting larval teleosts are unable to accommodate simultaneously high levels of growth and sustained activity (Wieser et al., 1988). Growth is critical during larval and juvenile phases of fishes, as smaller, slow growing individuals are more susceptible to predation (see Bigger is Better Hypothesis, Miller et al., 1988; Growth-Predation Hypothesis, Anderson, 1988). Aerobic power budgets, which account for how animals allocate energy to particular organ systems and behaviours, can explain compromises between growth and activity (Guderley & Pörtner, 2010). For example, the common roach (*Rutilus rutilus*) behaviourally reduces swimming activity during gonadal development (Koch & Wieser, 1983). During the onset of metamorphosis, growth hormone gene expression is significantly increased in some flatfishes (Vagner et al., 2013). In general, larvae must ingest more energy to meet increased metabolic demands; therefore, very little energy is stored, and most ingested energy goes to increasing muscle mass and into reproductive structures, unless swimming is absolutely necessary (Bishop & Torres, 1999; Parra & Yúfera, 2001; Guderley & Pörtner, 2010). In this study, despite a large decrease (30-40%) in U_{crit} after metamorphosis, aerobic scope remained the same across metamorphosis, suggesting that energy is simply allocated to other physiological processes (i.e., likely growth) over swimming, and a lower U_{crit} is a behavioural response rather than a physiological impairment (i.e., as would be seen with a decrease in aerobic scope or altered SMR and/or MMR). Additional ecological and behavioural studies focusing on post-settlement growth and survivorship in reef fishes, from the laboratory and field, will help support my hypothesis.

6.5.2.2. Growth, behaviour and ecology of reef fishes post-metamorphosis

High swimming performance and hydrodynamic processes are likely important for pelagic reef fish larvae when finding a suitable reef on which to settle and, based on the findings from **chapter 5**, require a lot of energy. The reefs they settle to present quite different challenges compared to the pelagic environment the larvae inhabited. The high mortality rates of newly settled reef fishes are mainly associated with size/growth-selective mortality (McCormick & Kerrigan, 1996; Searcy & Sponaugle, 2001; Hoey & McCormick, 2004) , and thus, finding a reef habitat with abundant resources is critical (Jones, 1986). Growth rates among metamorphosed juvenile reef fishes are generally faster than during their pelagic phase (i.e., based on otolith markings), as found in black-axil chromis (*Chromis atripectoralis*), neon damselfish (*Pomacentrus coelestis;* Thorrold & Milicich, 1990), French grunt (*Haemulon flavolineatum*; Brothers & McFarland, 1981), bluehead wrasse (*Thalassoma bifasciatum*; Victor, 1986), green damselfish (*Abudefduf abdominalis*; Radtke, 1985), Nagasaki damselfish (*P. nagasakiensis*), and Ward's damselfish (*P. wardi*; Pitcher, 1988). This is proposed as a mechanism

whereby fish can quickly "establish themselves" onto a reef and reduce predation by growing larger (Thorrold & Milicich, 1990). Several studies have shown the advantages of fast growth rates and good condition (e.g., protein and lipid reserves), which are qualities retained from their pelagic history as a larva, on survivorship post-metamorphosis (Searcy & Sponaugle, 2001; Hoey & McCormick, 2004; Grorud-Colvert & Sponaugle, 2006; . Juvenile reef fishes must balance energy use carefully, as wasted energy on swimming may compromise growth and increase predation risk.

Upon reaching a reef, metamorphosed juveniles exhibit typical neophobic behaviours – risk-adverse behaviours in response to novel stimuli – such as sheltering, to reduce predation risk of predation (McCormick et al., 2017). Recently metamorphosed fishes in good condition shelter more, as their endogenous reserves contribute more to growth than foraging (Grorud-Colvert & Sponagule, 2006). This has also been shown in juvenile Nassau grouper (*Epinephelus striatus*), as sheltering maximizes the relationship between growth rate and predation risk until larger sizes are achieved (Dahlgren & Eggleston, 2000). Indeed, post-metamorphosis life for reef fishes is largely focused on growing larger to reduce predation risk and to accommodate gonadal tissue (Liu & de Mitcheson, 2009). Investing energy into high swimming capabilities would compromise this. For example, sablefish (*Anoplopoma fimbria*) in good condition had lower U_{crit} than individuals in worse condition, as more energy was dedicated to tissue growth (Sogard & Olla, 2002). However, this must be finely balanced, as survivorship among fast-growing bicolor damselfish (*Stegastes partitus*) was lower than their slow-growing counterparts; increased metabolic demands associated with tissue synthesis occurred at the expense of tissue maintenance, and predation occurred due to increased foraging behaviour (Rankin & Sponaugle, 2014). Under intense predation pressure, it would be disadvantageous for metabolic rates to drastically change upon metamorphosis for any species of reef fish. Subtle changes in SMR may increase foraging behaviour to supply energetic demands, which would increase growth, but also increase the risk of being eaten. Therefore, a more efficient metabolic strategy for newly metamorphosed individuals would be to direct ingested energy toward growth rather than swimming, and behaviourally adjust their willingness to swim at high speeds. The swimming respirometry data from the current study are able to provide physiological support to the ecology (i.e., the importance of finding an ideal reef where growth would be maximized) and behaviours (e.g., sheltering vs. high foraging) of newly metamorphosed reef fishes. However, several studies have anecdotally highlighted the importance of the cellular mechanisms underpinning these changes during metamorphosis (McCormick et al., 2002; Nilsson et al., 2007a; Leis et al., 2011). Indeed, up until this point, I have discussed metamorphosis at the whole animal scale (i.e., oxygen uptake rates and swimming performance), within the context of habitat (i.e., reef or pelagic). I will now discuss changes at the cellular level and how such changes may influence swimming performance around metamorphosis.

6.5.3. Changes in muscle mitochondria volume density and consequences on whole animal performance

Many studies have hinted at the physiological changes coral reef fishes undertake during metamorphosis, yet to date, few studies have examined these changes empirically. While some studies have noted changes in activity levels upon metamorphosis (e.g., Stobutzki & Bellwood, 1994; Leis et al., 2011), no current studies have investigated whether properties of muscles change during this period to facilitate these changes in activity. Muscles are highly plastic tissues that rapidly change with external (e.g., hypoxia and temperature) and physiological (e.g., exercise and development) stimuli (Granata et al., 2016). Within 24 h of settlement, the red muscle tissue in both the pectoral and trunk fins experience an increase in mitochondria volume density (V_{mt}), but V_{mt} gradually decreased over a few days following metamorphosis. I initially hypothesized trunk V_{mt} to be: i) highest during larval periods, and ii) higher than pectoral muscle V_{mt}, particularly at the larval phase, because larvae rely on high amplitude body undulations. Given that Ambon damselfishes rely on pectoral fins for swimming as adults, and larval Ambon damselfishes are very well-developed at the end of their pelagic larval phase, pectoral swimming may be the primary mode of swimming used by these fishes before reaching the reef. However, the dramatic increase in V_{mt} in both pectoral and trunk muscle within 24 h of metamorphosis and the immediate impact this has on swimming efficiency (Cost of Transport; COT) suggest that rapid changes at the tissue level may have significant impacts on energetic demands for swimming. In the next few sections I will discuss the causes of mitochondrial proliferation, the role of the thyroid during metamorphosis, and how this may impact whole animal performance.

6.5.3.1 Causes for mitochondria proliferation

Mitochondria are casually referred to as the 'powerhouse' of the cell, and their role in oxidizing energy substrates to produce ATP (adenosone triphosphate) drives all energy demanding reactions within a cell, thus having a 'bottom-up' impact on tissue, organ, system, and whole-organism function (Harper & Seifert, 2008). Indeed, regulating mitochondria densities has important ramifications for oxidative capacities and energy demands of tissues, as well as how well tissues cope with oxidative stress (e.g., reactive oxygen species; ROS; Moyes et al., 1998; Hood et al., 2018; Heine & Hood, 2020). Mitochondria proliferation is beneficial in that there is an increase in ATP production, increase protein synthesis, increases in oxygen diffusion into cells (e.g., increased rate of oxygen transfer across gills), and decreases diffusion distances of substrates between mitochondria (Sänger, 1993; O'Brien, 2011). Mitochondria proliferation or mitochondria biogenesis is caused by increases in PGC-1α, which is a transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1α, and this is due to the increased expression of the PPARGC-1α gene (Urschel & O'Brien, 2008). Several stimuli can cause

an increase in the expression of the PPARGC-1α gene, such as endurance exercise, oxidative stress, nitric acid, and carbon monoxide (Sänger, 1993; Moyes et al., 1998; Hoppeler et al., 2002; O'Brien, 2011).

Mitochondria volume densities in young Ambon damselfishes increased 24 h post-metamorphosis and decreased back to larval V_{mt} within 72 h of settlement. During this transition period from the pelagic to reef habitats, several environmental stimuli (e.g., hypoxia) are present, and behavioural changes that would influence mitochondria biogenesis. The shift from pelagic (i.e., active) to a benthic (i.e., less-active) lifestyles has been found associated with decreased muscle V_{mt} in marine teleosts, as swimming becomes less of a priority in a benthic animal's energy budget (Johnston & Camm, 1987; Yamano et al., 1991). Decreasing exercise and performance has been found associated with lower muscle V_{mt} in other vertebrates as well, such as mammals (Moyes et al., 1998; Granata et al., 2016). Additionally, environmental oxygen levels influence mitochondria biogenesis. Generally, hypoxia suppresses ATP production and therefore, any aerobically driven activities, such as swimming, growth, reproduction, and basic tissue maintenance will also be suppressed (Sokolova, 2018). Reduced oxygen supply has been found associated with mitochondria biogenesis in mammals and fishes, as a mechanism to increase oxygen extraction from blood during exposure to oxygen limiting environments (Van Ekeren et al., 1992; Bassett & Howley, 2000; Sokolova, 2018). Some animals reduce activity levels during hypoxia events to conserve energy (Nilsson et al., 2007a,b). Indeed, reef fishes experience changes in exercise levels and experience hypoxia events as they metamorphose onto the reef. While it is reasonable to hypothesise that, after a pelagic period, where there is an intense need for swimming, mitochondria proliferate and then deteriorate as fish behaviourally choose not to swim (e.g., increase sheltering behaviour, described in 6.5.2 of current study), this hypothesis is unlikely supported, as V_{mt} in both measured muscle types of larval Ambon damselfishes are the same as in 48- and 72-hour post-metamorphosis juveniles. Similarly, an increase in muscle V_{mt} may be a mechanism to tolerate nocturnal hypoxic events that occur on the reef due to coral respiration (Nilsson et al., 2007a,b). However, since these events occur daily, it would be assumed that mitochondria would elevate upon metamorphosis and remain elevated into late juvenile and adult phases to cope with chronic hypoxia exposure. The decrease in muscle V_{mt} may suggest that another mechanism is in place to allow reef fishes to tolerate hypoxia (see Nilsson et al., 2007a,b). Mitochondria are energetically expensive to produce, and high volumes are energetically taxing to maintain. Given that activity levels decrease at settlement, elevated mitochondria levels beyond 24 h would require additional energy, either from reserves or foraging. Therefore, new recruits would need to be in good condition; otherwise, they would risk predation to maintain high energy levels. While activity changes and hypoxia may contribute to the observed increase in V_{mt} in both pectoral and trunk musculature, it is possible increases in V_{mt} are caused by changes in thyroid hormone levels associated with metamorphosis.

6.5.3.2. Thyroid hormones and their role in metamorphosis of fishes

The thyroid gland, an integral part of the endocrine system, produces thyroid hormones (TH), which have a wide array of functions in animals, such as regulating circadian rhythms in vertebrates, catabolism of sugars and fats, and thermoregulation in mammals (summarised in Besson, 2017). They are also notably involved in developmental processes involved in metamorphosis, with the classic example being the large restructuring in anatomy and of amphibians as they transition from tadpoles to frogs (summarised in Besson, 2017). While initially believed to only regulate flatfish metamorphosis, it is now well established that thyroid hormones, in combination with environmental cues, universally play an integral role in metamorphosis in teleosts, including reef fishes. In teleost fishes, TH mediates morphological changes and ontogenetic habitat shifts (Inui & Miwa, 1985; Perez et al., 1999; Trijuno et al., 2002; Shiao & Hwang, 2006; Chin et al., 2010; McMenamin & Parichy, 2013; Holzer et al., 2017; Campinho, 2019; Besson et al., 2020). Prior to metamorphosis, TH levels in teleost larvae are near if not at zero (Matsumoto & Tanaka, 1996; Campinho, 2019). The thyroid gland produces, mainly, thyroxine (T4), which transforms into its active form, triiodothyronine (T3) (Holzer et al., 2017; Campinho, 2019). Both T4 and T3 are at peak concentrations during metamorphosis (Campinho, 2019), and then both decrease back to larval levels within 24-72 h post-metamorphosis (Matsumoto & Tanaka, 1996; Klaren et al., 2008; Holzer et al., 2017). However, during the period surrounding metamorphosis the influence that thyroid hormones have on whole-animal and cellular physiology, particularly activity, is also critical.

Several studies have noted increases in activity patterns in metamorphosed fish, which has been related to increased thyroid hormone levels. Indeed, increasing TH levels are related to increasing swimming performance in, such as in cod (*Gadus morhua*), as they regulate metabolic enzymes in the brain, liver, and skeletal muscles that lead to increases in energy production and aerobic capacity (Edeline et al., 2005). More specifically, this relationship between TH and activity is due to the role thyroid hormones play in regulating PGC-1α expression. As previously mentioned, PGC-1α regulates mitochondria biogenesis and therefore stimulates oxidative pathways in muscles allowing them to uptake more oxygen (Irrcher et al., 2003; Harper & Seifert, 2008; O'Brien, 2011; Bloise et al., 2018). Indeed, TH activates mitochondria biogenesis, which would increase muscle function (Little & Seebacher, 2013). Since TH increase in concentration during reef fish metamorphosis (i.e., within 24 h of settling onto a reef), I would predict that the proliferation of mitochondria measured in 24 h metamorphosed juvenile Ambon damselfishes may be an artefact due to an increase in circulating TH levels. This may be why V_{mt} decreased back down to levels exhibited by larvae, as TH levels would be predicted to decrease during the intial 24 h period in which metamorphosis is taking place. While TH levels decrease within 24 h of metamorphosis for most teleosts (e.g., flatfishes), in some reef fishes, like blue-green chromis (*Chromis*

viridis), TH levels reduce to zero by 72 h post-metamorphosis (Holzer et al., 2017). Measurements of TH levels would support my prediction.

In general, the correlation between how cellular processes translate to whole-animal performance is still not very well understood (Heine & Hood, 2020). A valuable physiological metric to correlate changes at the tissue level (e.g., muscle mitochondria volume density) to performance is Cost of Transport (COT), which is the energy required to move an animal a unit distance (Schmidt-Nielson, 1972). Cost of Transport has been used to evaluate the amount of energy require to support locomotion, and determine optimum swimming speeds for a wide variety of species such as skates (e.g., *Raja eglanteria*; Di Santo et al., 2017), kingfish (e.g., *Seriola lalandi*; Palstra et al., 2015), birds (Hedenström & Alerstam, 1995), and marine mammals (Williams, 1999). Understanding the amount of energy required for movement at a given speed provides a better estimation as to how much of an animal's energy budget is being directed for movement. Additionally, optimal movement speed (i.e., the fastest speed with the lowest energy cost) has intrinsic ecological relevance, as the speeds encountered in the natural environment are therefore related to biological fitness of the species. For recently settled coral reef fishes, oxygen uptake is critical to support high growth rates so that fish can establish themselves onto the reef, mitigate predation risks (see *6.5.2.2* for details), and to swim to find an ideal patch of reef to call home.

I found a correlation between V_{mt} in pectoral muscles and Cost of Transport, as COT was lowest during the exact periods where V_{mt} in pectoral muscles was the highest. This may suggest that muscle tissue V_{mt} regulation, perhaps via TH, during the onset of metamorphosis allows reef fishes to be more efficient swimmers when they reach a reef, thus providing a brief, 24-72 h window for the fish, without expending too much energy, to find an ideal patch of coral on which to settle. However, it is important to note that correlation does not necessarily mean causation, and future studies should investigate whether genes that code for mitochondria proliferation are active during this period. Regardless, given the importance of available energy stores to post-metamorphosis growth, a reduced COT may provide newly metamorphosed reef fishes, particularly those in good condition, a distinct advantage during such a critical survival bottleneck in their life history, as there may be more energy available to support growth rates. To my knowledge, no other studies have investigated the relationship between V_{mt} in different muscles and whole animal performance (i.e., oxygen uptake rates during activity) in any teleost larvae or juvenile fish species. This is critical information, especially for reef fishes, as metamorphosis is an energetically taxing process, and given the multitude of pressures larval fishes already face (e.g., reef predators and tidal energy), being energy efficient in terms of swimming may improve survival.

6.5.4. Conclusions

Metamorphosis is a critical, and complicated stage in reef fish life history. The shift from pelagic to reef habitats means larval reef fishes must endure drastic changes in environmental conditions (e.g., temperature), current/wave energy, stimuli (e.g., reef noise), sources of energy, and predators. Amazingly, reef fishes endure these changes within 24 h. Behavioural and anatomical modifications have been noted in previous studies (e.g., McCormick & Makey, 1997; McCormick, 1999; McCormick et al., 2002, 2017; Leis et al., 2011), and in the current study, I have provided empirical evidence to suggest that there are also physiological changes prior to and upon metamorphosis. It is likely that swimming decreases as a behavioural response to regulate growth rates, as swimming is an energetically expensive task. Aerobic energy budgets are maintained during metamorphosis, as a hypothesized strategy to allow newly metamorphosed fishes to reduce foraging costs and rely on endogenous energy reserves for growth. This highlights the importance of body condition and larval history on successful juvenile survivorship (Hoey & McCormick, 2004; Gagliano et al., 2007). Interestingly, muscle mitochondria volume densities in both trunk and pectoral tissues increase within 24 h of metamorphosis. At this time, there is also a decrease in the cost of transport, meaning reef fishes are more efficient swimmers upon reaching a reef. I hypothesized that the mitochondrial proliferation observed here is associated with increases in thyroid hormone levels and that mitochondria densities would be expected to dissipate as thyroid levels subsequently decrease. This area clearly warrants further investigation.

Taken together, while reef fishes experience many challenges upon reaching a reef, behaviourally adjusting their swimming patterns, maintaining aerobic energy budgets to maximize growth rates, and utilising the window of opportunity while cost of swimming is lowest, may facilitate successful transitions to a reef form the pelagic realm. Future work should focus on increasing the taxonomic range of study species (e.g., beyond pomacentrids) to determine patterns in oxygen uptake rates during exercise and whether changes in muscle mitochondria are universal among reef fishes. Measuring growth and thyroid hormones during metamorphosis would also provide conclusive evidence as to whether the hypotheses regarding energy the relationship between allocating energy for growth post-metamorphosis and mitochondria biogenesis is related to thyroid levels can be supported. The addition of more physiological information to metamorphosis may reveal how reef fishes make this transition within such a short window of time, importantly, supporting recruitment and future adult population structure and viability of reef fishes.

Chapter 7. General Discussion

The high swimming performance observed in the early life stages of tropical coral reef fishes has captivated the attention of marine scientists since the early 1990s (Stobutzki & Bellwood, 1994; Leis, 2006). The ability for an animal, ranging in size from ≤1-3 cm in body length, to swim 20 to 100 cm s^{-1}, albeit species-specific, is an amazing feat of athleticism among vertebrates (Fisher et al., 2005). Understanding how reef fishes swim to such capacities as late-stage larvae cannot be solely investigated using one subdiscipline; thus, this topic requires the integration of numerous levels of biological organization, methodological techniques, and a comprehensive synthesis of the current literature to begin to understand the development of these 'miniature athletes'. This thesis investigated athletic performance in larval fishes by integrating together different methodological techniques (e.g., critical swimming speeds, endurance swimming, *in situ* observations) used to swim larval fishes (**chapter 2**), morphological (e.g., body shape, muscle area) and environmental (e.g., habitat association, water temperature) factors that influence performance, while considering species relatedness through phylogenetic comparative analyses (**chapter 3**). Moreover, changes in oxygen demands and swimming speed over the entire larval duration of a reef fish were examined, and changes in gene expression for proteins coding oxygen transport and storage were investigated (**chapter 5**). Finally, changes in energy demands and muscle properties that allow fish to successfully recruit to reefs were also investigated (**chapter 6**). This integrative approach allows for a better mechanistic understanding as to how reef fishes support swimming performance across ontogeny, which is key to understanding connectivity among reef fish populations.

7.1 Swimming and developing in the open ocean

The primary goal of the larval phase for any teleost fish species is to develop tissues and organ systems and to grow quickly so that they can swim well and mitigate interactions with predators (Downie et al., 2020). Much of the early larval fish biology research that investigated swimming performance focused on temperate, commercially important species, such as cods, herrings, and flatfishes (Miller et al., 1988). Throughout most of their larval phase, fish larvae are generally poor swimmers and, as such were considered planktonic passive particles that had no influence on where they ended up in the marine environment (Roberts, 1997). Pioneering laboratory studies (Stobutzki & Bellwood, 1994), *in situ* observations (Leis et al., 1996), and protocols using genetic markings (Jones et al., 1999) have shown that tropical reef fishes, particularly at the stage of settlement, are capable of incredible swimming speeds, which implies that this behaviour is important for recruiting to new reefs (Almany et al., 2017) and/or recruiting back to natal reefs (Jones et al., 2005). Placing swimming within the proper ecological context requires use of appropriate swimming methods (**chapter 2**).

When viewed in relation to other groups of fishes, tropical reef fishes are capable of fast critical swimming speeds (U_{crit}), thus enabling them to swim against currents (e.g., Fisher et al., 2005), *in situ* speeds that are generally faster than ambient currents (Leis et al., 1996), and they are capable of swimming for long distances (i.e., >20km), especially when provided food (Fisher & Bellwood, 2001) (**chapter 3**). Interestingly, temperate reef fishes are also capable of swimming at relatively high speeds (Leis et al., 2012, Failletaz et al., 2018), and it is of interest to compare swimming performance amongst all larval fishes – where data are available – to provide a broader understanding of larval fish performance across latitudes. Indeed, there may be more than just latitude, as tropical reef fishes are capable of faster critical swimming speeds and faster *in situ* speeds than tropical pelagic and non-reef demersal species at a common temperature (**chapter 3**). Additionally, temperate reef fishes swimming in 10°C cooler water do so within a similar capacity as tropical pelagic and non-reef demersal fishes. Temperate reef fishes also exhibit the fastest increase in growth when compared to all other groups of fishes, regardless of latitude (**chapter 3**). Therefore, the need to find and settle on a coastal reef may be one of the key drivers of swimming performance among teleost fishes at the larval stage, and aspects of their morphology may support this performance.

Morphology is generally a good indicator of fish performance (e.g., Fisher & Hogan, 2007), and reef associated species generally exhibit a more robust shape with larger muscle blocks than pelagic and demersal fishes (**chapter 3**). Clearly, properties of muscle tissues (e.g., muscle mitochondria volume densities, as investigated in this thesis), metabolic enzyme activities, and oxygen demands are different for reef fishes than other groups of fishes that either remain in pelagic habitats or select a random benthic habitat in which to settle upon (**chapter 3**). Greater muscle tissue synthesis allows fish to achieve faster swimming speeds but relies on oxygen to power associated protein synthesis. Tropical species generally have shorter larval durations than temperate species, and therefore must synthesise muscle tissue within a narrower timeframe (**chapter 2**). While seldom performed (<5% of studies, Peck & Moyano, 2016), previous work on oxygen uptake rates during activity has shown that settlement stage tropical reef fishes have the highest oxygen uptake rates of any teleost fish species (Nilsson et al., 2007a). This high oxygen uptake rate is likely an artefact of living in warmer waters but is a key contributor to high swimming performance and muscle tissue synthesis (**chapters 2 and 3**). Several challenges, including technological limitations (e.g., small enough sensors to measure changes in oxygen) have precluded a clear understanding as to the energy budgets and oxygen requirements larval fishes need to support swimming performance (Peck & Moyano, 2016). Altering the design of the swimming chamber (e.g., adding inserts that greatly reduce respirometer volume) and fiberoptic oxygen sensors have contributed significantly to reliable measures of oxygen uptake rates from larval reef fishes throughout development (**chapter 5**). As a result, my thesis shows that estimates of active metabolic rates of larval anemonefish (*Amphiprion*

melanopus) are significantly higher than the oxygen uptake rates of other reef species (e.g., *Pomacenetrus amboinensis, Chromis atripectoralis;* Nilsson et al., 2007a) (**chapter 5**). These high oxygen uptake rates likely support high growth rates and consistent increases in U_{crit} on a daily basis (**chapter 5**). While many species are found to increase growth and swimming in daily increments, these costs may be higher for anemonefishes, and other direct developing fishes, as direct developing fishes hatch with well-developed, yet metabolically demanding tissues, such as eyes, brain, and muscles (Roux et al., 2019). While these tissues provide larvae with advantage earlier in life (e.g., better vision and swimming capabilities), the large energetic costs may mean these larvae are more susceptible to mortality from starvation or environmental change (Killen et al., 2007; see following section for details). Taken together, however, it is possible that such high oxygen uptake rates enable reef fishes to achieve large robust body morphologies within a short larval duration (**chapters 2, 3, 5**), which will have critical ecological consequences for finding a suitable reef.

7.2 Reef-ward bound: preparing for the reef and energetics of recruitment

The high swimming performance of reef fishes may confer a myriad of advantages, but none more important than being able to find and select on a reef. Oceanic and reef currents are distinct in their intensity, and the high critical swimming speed and *in situ* speeds of reef fish larvae are generally adequate for them to swim against such currents (**chapter 3**). Select groups (i.e., those where information on U_{crit} and *in situ* swimming is available) of tropical pelagic and non-reef demersal species are not capable of overcoming such currents in shallow parts of a reef (e.g., those around Lizard Island), but their swimming capabilities may suffice in deeper areas (**chapter 3**). Placing high performance swimming (**chapter 3**) and the oxygen uptake demands required of swimming (**chapter 5**) within an ecological context is important, as the physiology of an animal can then be linked to how the animal responds to and contributes within its habitat. Moreover, a physiological approach across various life stages throughout early development is also important, as different life stages may experience different stressors and energetic demands from their habitats. This is especially true for animals with a bipartite life history, such as reef fishes. As such larval reef fishes developing in the open ocean become specialised to pelagic life (e.g., strong athletic swimming against ocean currents), but must make a transition and quickly become specialised to a reef which has its own distinct challenges, notably nocturnal hypoxia events.

Coral reefs can experience nocturnal hypoxia events due to coral respiration (Nilsson et al., 2007a,b). Adult and juvenile coral reef fishes are tolerant to hypoxia (Nilsson et al., 2007b); however, hypoxia tolerance does not immediately develop in fishes upon reaching the reef. There is a generalisation that hypoxia tolerant species have low activity levels and lower oxygen uptake rates than hypoxia intolerant species (Nilsson, 1996). However, reef fishes have exceptional swimming performance and

high oxygen uptake rates as larvae (**chapter 3, 5**); therefore, the transition larvae make to become a hypoxia-tolerant, benthic animal must happen at some stage in the pelagic environment, before the larvae reach the reef. I noted a significant decrease in oxygen uptake rates near the end of the larval duration of the anemonefish. This decrease was complimented by increases in gene expression of embryonic haemoglobin (which has a high affinity for oxygen), and myoglobin, which is used for oxygen storage and diffusion into tissues. Moreover, there were also increases in gene expression for cytoglobin and neuroglobin, which are key for oxygen storage in connective tissues and brain, respectively (**chapter 5**). The increases in expression of these genes in conjunction with lowered estimates of metabolic rates suggest that the larvae are preparing for a transition to a hypoxic environment, whereby delivery of oxygen to tissues is vital to prevent tissues from being starved of oxygen (Brownlie et al., 2003). Simultaneously, the increase in expression of genes responsible for oxygen transport may also support high swimming performance at this stage (Brownlie et al., 2003), where the larvae will need to swim fast, against reef and ocean currents, in order to settle. These conclusions are still preliminary, since these genes and their functions have been described for zebrafish, but not yet for anemonefishes. However, the changes at the gene expression level in relation to alterations in whole animal performance (i.e., oxygen uptake rates and swimming performance) provide compelling evidence that significant physiological changes are occurring during the larval phase to support hypoxia tolerance and possibly high swimming performance as well. Upon settling onto the reef as a juvenile, performance generally decreases.

Several studies have noted significant decreases in swimming performance of larval reef fishes as they reach a reef and metamorphose into a juvenile (Stobuzki & Bellwood, 1994; Leis et al., 2011). The transition to a reef marks a critical developmental milestone in reef fish life history, characterised by large mortality from reef predators (McCormick et al., 2002). Therefore, this transition must occur quickly, so juveniles can establish themselves onto a reef. How energy demands change during activity surrounding this event has yet to be investigated. Ambon damselfishes were found to decrease their swimming performance by 40% within 72 hours of settlement (**chapter 6**), following a similar pattern as previous studies (e.g., Stobutkzi & Bellwood, 1994; Leis et al., 2011). This decrease in performance did not result in any associated changes in oxygen uptake rates, suggesting that i) larvae may choose not to swim (behaviourally mediated; Faria & Goņalves, 2010; Silva et al., 2015) and may divert energy toward growth to reduce predation pressures (Gagliano et al., 2007; Hoey & McCormick, 2004) and ii) hypoxia tolerance may limit energy requirements for performance due to the opposing demands required of high swimming capacity and hypoxia tolerance (Nilson, 1996). Interestingly among this decrease in swimming performance upon settlement, I found a 20% increase in muscle mitochondria volume density, which was related to a decreased Cost of Transport (i.e., the energetic cost to move a unit distance) for up to 48 h post settlement (fish are swimming more efficiently within the periods of 24-48h post-settlement; **chapter**

6). Given the demands placed upon newly settled reef fishes, this temporary decrease in swimming costs may relax the energetic demands of growth and may also give newly settled reef fish a 'window of time' to find an ideal reef patch at a reduced energetic cost. Similar to the findings of **chapter 5**, significant physiological changes occur at multiple levels of biological organization (i.e., whole animal to cellular) upon settlement. These changes clearly have ramifications for ecological processes including settlement and recruitment to adult populations. Incorporating a physiological framework, such as the information required to identify these 'windows of time' will aid not only in understanding the dynamics of reef fish populations but will also help in better reef conservation and management.

7.3 Implications for management and swimming in an uncertain world

Physiology experiments generally include statements as to how findings can be applied to conservation and management of particular species; however, that link is generally not fully realised. Conservation physiology, defined as 'the study of physiological responses of organisms to human alteration of the environment that might cause or contribute to population decline' (Wikelski & Cooke, 2006) is an emerging field of study interested in taking a mechanistic approach using a variety of tools to understand conservation problems (Cooket et al., 2013, 2020). These tools scale from genetic to whole animal, to ecosystem function to better understand how alterations in environmental conditions change the physiology and subsequently the behaviour of animals (Cooke et al., 2013). My thesis takes an integrative approach utilising a wide range of tools examining the genetic, cellular, and whole animal processes underpinning swimming performance in reef fishes. The applications of my research are important for management of reef fishes, given the relationship between behaviour and physiology. Simply put, swimming behaviour of reef fish larvae that influences dispersal patterns in the ocean and further supports larvae successfully transitioning to a reef to support adult populations is controlled by physiological processes at the cellular (**chapter 6**), molecular (**chapter 5**), and whole animal levels (**chapter 3, 5, 6**). Why does this matter and how does swimming performance of reef fish larvae contribute to reef conservation?

Coral reefs globally are under unprecedented threat from climate change (Hughes et al., 2018; Hughes et al., 2018). Coral reef health relies of reef fishes and their functions. For example, rabbitfishes consume algae that compete with coral (Hixon, 1997), parrotfishes scrape away algae facilitating settlement for larval corals (Bonaldo et al., 2014), triggerfishes consume coral-consuming animals (McClanahan, 2000), predators, such as coral trout (e.g., *Plectropomus leopardus*) structure reef fish populations (reviewed by Frisch et al., 2016). Specifically, *P. leopardus* is not only an important meso-predator on reefs, but also a highly valued fishery species ($67 million Australian dollars per annum), supporting sustenance and sport fisheries (Pratchett et al., 2013). Future populations of these important

groups, among several others, rely on a consistent supply of larvae. Despite the wide diversity of reef-associated fishes, including the ones mentioned, the vast majority of species have the same life history pattern as the fishes used in my thesis. Therefore, dispersal, connectivity and recruitment that supports future populations rely on swimming behaviour as larvae and the physiological processes, described in my thesis, supporting them. From a management perspective, understanding the physiological processes (e.g., oxygen uptake rates) that support larval dispersal patterns and recruitment to reefs is important for predicting future population structure. While my thesis has set the foundation for understanding these processes, I will now highlight how anthropogenic stressors may limit larval reef fish performance and the consequences this may have on dispersal and recruitment patterns.

The high-capacity swimming of larval reef fishes is supported by their unique physiological and morphological attributes, as discovered in this thesis, such as high oxygen uptake rates, robust body morphologies, and changes in the expression of genes related to oxygen transport. These attributes facilitate the reliable dispersal patterns observed in nature among reef fish populations (Almany et al., 2017). While the degree by which reef fishes utilise this high swimming capacity is still an area of ongoing research, the incorporation of behaviour and associated physiological support mechanisms into dispersal models may better predict recruitment events and how energetic demands during larval stages may be impacted by local stressors (e.g., weather patterns, heat waves, etc.). However, it is to be determined how dispersal patterns will change under anthropogenic climate change. Reef fish larvae have high oxygen uptake rates to function, yet have a very narrow aerobic scope (**chapter 5**). From a conservation physiology perspective, this means they may have difficulties 'physiologically multi-tasking' between growth, development, swimming, and responding to environmental change (Killen et al., 2007). Therefore, subtle changes in environmental conditions may either result in mortality or less energy available for growth, development, and swimming that could have larger implications for dispersal potential. To date, no studies have investigated the impact of climate stressors on dispersal potential (e.g., endurance tests) of any fish species during their larval phase (**chapter 2**). Such studies would provide managers critical information regarding changes in abundances of local annual recruitment, alterations in genetic diversity due to changes in dispersal between populations, and future stock structure under current and future warming scenarios. Regardless, information from the current thesis can provide valuable physiological information within the context of dispersal, connectivity, and recruitment of reef fishes. Many of these applications directly relate to the goals of conservation physiology (Cooke et al., 2020) and improving management of reef ecosystems.

7.4 Future directions for research

While this thesis initiated many investigations and has drawn several conclusions regarding the physiological aspects of swimming performance of larval reef fishes, this thesis has also sparked many additional questions for further studies. Many of these questions are highly relevant to the field of conservation physiology. One aspect of **chapter 2** involved investigating different environmental stressors on reef fish performance. Very few studies have swum larval reef fishes under future ocean conditions relevant for climate change. Additionally, most studies investigate how these climate change stressors will influence later larval stages (i.e., at post-flexion). One of the take-home messages of this thesis is the importance of investigating how a trait changes over the entire larval duration of a fish's early life history, not just at later life stages. Future studies should investigate the impact of ocean warming, ocean acidification, reductions in prey availability, toxicants, and combinations of these stressors on early larval stages (i.e., newly hatched through to flexion stage) in addition to post-flexion stages, as the earlier life stages are likely more vulnerable to these stressors. Indeed, the recent increased frequency of marine heatwaves, ongoing ocean warming and acidification, and pollutants may have a more detrimental impact on pre-flexion stages, which would have substantial bottom-up effects on fish population structure and the viability of future stocks.

Chapter 3 utilised the current larval fish swimming literature for the analyses, which revealed a general high bias toward Percomorpha fishes. When comparing communities between different biogeographical regions (e.g., latitudinal differences, in this case) the structure of those communities will vary quite considerably. Tropical latitudes have a higher community composition of primarily reef associated species, such as butterflyfishes, damselfishes, surgeonfishes, and triggerfishes, among many other speciose taxa that are not found at temperate latitudes. These biases have been recognised when discussing the results of **chapter 3**. **Chapter 2** compared swimming performance of Percamorpha fishes and Clupeiforme fishes across latitudes and found similar swimming performance between the groups. Comparing swimming speeds of taxa that overlap between communities that vary in latitude will provide better empirical evidence as to why swimming differs between groups of fishes and the factors influencing such profound differences in swimming performance. This will help further test my initial hypothesis from **chapter 3** that habitat association influences larval swimming performance, specifically the need to find an ideal patch of reef requires different swimming capabilities than remaining in the pelagic environment.

The changes to the larval swimming respirometer design will open up the possibilities of further studies on energy demands of larval fishes during exercise. Besides expanding taxonomic diversity (e.g., fishes that are pelagic spawners versus direct developers) and swimming larval fishes under different

environmental conditions and upon exposure to various other anthropogenic stressors (as suggested above for **chapter 2**), the addition of physiological information to individual based models for dispersal patterns will likely be the most prevalent application of this chapter and for moving forward in the field of larval fish exercise physiology. I swum my fishes using a stepped velocity protocol to elicit a fatigue response so I could measure aerobic scope. Aerobic scope is a valuable measurement, but it may be more valuable to swim larval fishes and measure oxygen uptake rates at ecologically relevant flow velocities (e.g., current flows they would experience in nature) for prolonged periods of time (e.g., a 200-minute endurance test; Downie et al., 2021b). This would provide valuable information regarding the oxygen demands that support long distance swimming between reefs connected by larval dispersal. Consequently, swimming larvae under future environmental conditions, such as those relevant to climate change, would predict how energy demands would change and whether this would have an impact on dispersal distances.

In both **chapters 5 and 6**, I attempt to relate changes at the molecular (changes in gene expression for haemoglobin, myoglobin, cytoglobin, and neuroglobin) and cellular level (changes in mitochondria volume density) with changes in whole animal performance (swimming speed, oxygen uptake rates, and cost of transport). Excluding my thesis, this has yet to be done on larval fishes, and in general the relationship between whole animal performance and changes in mitochondria and gene expression are widely unknown across vertebrate taxa. For this reason, understanding these relationships are compelling and have a diverse applicability for furthering our understanding of animal development, how genes and cells change under different environmental conditions, and how genes and cells support locomotion. Simply, we need more studies that measure both whole animal performance (oxygen uptake rates and locomotion) and the regulation/expression of associated genes and cellular processes. Specifically, for reef fish larvae, changes in gene expression and mitochondria volume densities may help support swimming performance relevant for dispersal among reefs, but more studies investigating a wider range of genes (e.g., genes associated with Krebs cycle regulation and mitochondria biogenesis) may further our understanding of the mechanistic link between performance within the animal. Additionally, the magnitude by which genetic and cellular processes change under climate change scenarios may help predict how performance and dispersal potential of reef fishes and whether they possess the adaptive capacity to react to these changes.

7.5 Concluding remarks

How do coral reef fishes develop into athletes? Reef fishes live on the physiological extreme, requiring high amounts of oxygen to support swimming, development, and growth. However, to fully answer this question I had to take a multi-disciplinary approach in my thesis, integrating together phylogenetics, swimming physiology, cellular processes, and gene regulation. Over evolutionary history,

tropical reef-associated species (and predictably temperate reef fishes as well) developed physiological mechanisms that enabled them to seek out and find a patchily distributed reef habitat after enduring periods of time out in the ocean as a larva. While their swimming capabilities have been noted, at least since the last decade of the 20th century, my thesis emphasises that high oxygen uptake rates over early ontogeny contribute to fast growth rates and the 'athletic' swimming speeds. My thesis also highlights the critical changes at the cellular and molecular levels that may predictably contribute to alterations in how oxygen is transported and delivered to tissues, how this may prepare larvae for the hypoxic environment on reefs, and the rapid alterations in muscle function that facilitate recruitment processes. Indeed, tackling the questions of this thesis would not have been possible without taking a multi-disciplinary approach with the aim to integrate different levels of biological organization. That said, the questions that have arisen because of my thesis will require a similar, integrative approach. Regardless, investigating the processes responsible for the high swimming performance among reef fish larvae will provide valuable insight into the role their unique physiological plays into dispersal, connectivity, and recruitment processes. While studies conducted in the early 1990s that first discovered the high-capacity swimming of reef fish larvae piqued the curiosity of marine ecologists, the advancements in understanding the physiology of such performance will hopefully continue to captivate the scientific community to further our understanding of these amazing miniature athletes.

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