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The effect of parental and embryonic predator environments on offspring



Thesis submitted by
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Statement on the Contribution of Others

This thesis includes collaborative work with my primary supervisor Prof. Mark McCormick, as well as Kine Oren. My other supervisors Dr. Ashley Frisch and Prof. Geoff Jones provided guidance in initial discussions while determining the direction of my thesis, and Dr. Ashley Frisch provided editorial assistance. While undertaking these collaborations, I was responsible for research concept and design, data collection, analysis and interpretation of results. My co-authors provided intellectual guidance, editorial assistance, financial support and/or technical assistance. Financial support was provided by the ARC Centre of Excellence for Coral Reef Studies (M. McCormick), the James Cook University Graduate Research Funding Scheme and the College of Marine and Environmental Sciences, James Cook University. James Cook University provided laboratory space at the MARFU aquarium complex.

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General Abstract

Determining the role that predation plays in population and community dynamics is vital for understanding complex ecosystems, such as coral reefs. The presence of predatory species often varies greatly with both space and time, and as such, prey species need to be able to rapidly learn and adapt to a variety of constantly changing threats. Using chemical and visual stimuli, individuals can not only identify relevant predators, but also react to them in a graded manner, depending on the level of risk they represent. Considerable research has focused on predator prey relationships and how they influence population dynamics on coral reefs. Yet, to date, no one has studied the role parental effects or the olfactory capabilities of embryonic reef fishes play in the identification of predators by prey. Therefore, this study examines the impact of predator presence and perceived risk, by both parents and developing embryos, on offspring in coral reef damselfishes.

Parental effects involve non-genetic (i.e., phenotypic) inheritance of traits, which can affect offspring development and behaviour. Previous research has shown that parental exposure to predation risk can both benefit offspring (e.g., increasing antipredator behaviours), or have maladaptive consequences (e.g., metabolic and functional disorders), effects which appear to be context and species dependent. However, none of this research has shown the transferral of specific predator information, nor identified the existence of transgenerational predator recognition. Hence, **Chapter 2** investigated whether a common coral reef damselfish, *Acanthochromis polyacanthus*, was able to transfer their learned recognition of a predatory threat in their environment to their offspring via parental effects. Breeding pairs of *A. polyacanthus* were exposed to one of three visual and olfactory treatments: predator, herbivore and saltwater control. Increases in heart rate induced by the

introduction of test odours demonstrated that the resultant embryonic offspring from the predator-treated parents reacted significantly more (almost twofold) to the parental predator than offspring from the other two treatments. Results also showed that the embryos were able to differentiate between the five test cues, showing innate recognition of threat odours, rather than a neophobic response. This chapter provides the first example of the transfer of specific predator information via parental effects in any species.

Embryos of amphibian species have been shown to not only detect olfactory stimuli, but also use such cues to learn predatory threats before hatching. Thus, the next step in the study was to determine whether damselfish embryos could learn novel predator cues using associative learning (**Chapter 3**). Using the clownfish, *Amphiprion melanopus*, I conditioned embryos with a combination of a novel predator odour and a conspecific alarm cue. By quantifying reactions as changes in heart rates, I showed that individuals that were conditioned learned to identify the predator odour as a threat; the cue elicited an increase in heart rate that was almost double that of the pre-conditioning response. Additionally, I showed that the closer to the expected time of hatching, the larger the increase in heart rate induced by conspecific alarm cues. These findings suggest threat cues also play a vital role in early life stage anemonefish, which are already known to imprint on odours in their natal habitat.

Predator-induced mortality rates are highest in early life stages; therefore, early recognition of threats can greatly increase survival chances. Some species of coral reef fishes have been frequently found to recruit back to their natal reefs. In this instance, there is a high chance of juveniles encountering their siblings, amongst other kin, after hatching. Kin recognition plays an important ecological role in that it allows individuals to protect their relatives and gene pool, and hence increase their inclusive fitness. Additionally, research has shown that affiliating with kin can enhance predator avoidance. Consequently, **Chapter 4** investigated whether two species of damselfish,

with differing life histories, recognised kin through their damage-released alarm cues. Results showed that both *A. polyacanthus* and *A. melanopus* can distinguish between their kin and other conspecifics, reacting more to alarm cues produced from the former. They also reacted more to cues from conspecifics than more phylogenetically distant heterospecifics. Early recognition of kin and cues from phylogenetically similar heterospecifics could decrease predator-induced mortality through cooperation with kin and/or avoiding predation through informed habitat selection.

Predatory threats can vary markedly with changes in habitat and ontogeny, and individuals will continually experience new cues throughout their lives, especially in biodiverse habitats like coral reefs. The threat sensitive hypothesis states that individuals should show a stronger response to cues that represent greater risk. As such, **Chapter 5** aimed to establish whether *A. polyacanthus* reacted in a threat sensitive manner to cues derived from conspecific donors from different life stages. This hypothesis was based on the premise that embryos and adults would be preyed upon by different species, due to predator gape limitations, rendering adult alarm cues less relevant than those from closer ontogenetic stages. Experiments found that *A. polyacanthus* embryos reacted in a graded manner, with embryo alarm cues eliciting a greater increase in the heart rate of embryos than damage-cues from juveniles or adults. Responding to damage-released cues based on the level of threat they represent can enable prey to prevent unnecessary energy expenditure avoiding predatory species that pose little or no threat. Conversely, if individuals deem a cue indicative of a threat that is not relevant to their particular life stage, this would incur energetic costs.

This research demonstrates that embryonic damselfishes have very well-developed olfactory capabilities that they can use to recognise predators and/or chemical alarm cues of both conspecifics and heterospecifics before hatching. Furthermore, this recognition can be augmented with parental information and/or individual experience

and learning. The existence of such refined mechanisms for identification of threats in the earliest life stages of the study organisms suggests that they serve a vital role in the chemosensory recognition of predatory threats.

Table of Contents

Statement on the Contribution of Others	i
Acknowledgements	ii
General Abstract	iv
Table of Contents	viii
List of Tables	x
List of Figures	xi

Chapter 1: General Introduction	1
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Chapter 2: Parents know best – conveying predator information to offspring through parental effects	16
2.1 Summary	16
2.2 Introduction	17
2.3 Methods	20
2.4 Results	24
2.5 Discussion	27

Chapter 3: Active in the sac – damselfish embryos use innate recognition of odours to learn predation risk before hatching	33
3.1 Summary	33
3.2 Introduction	34
3.3 Methods	36
3.4 Results	40
3.5 Discussion	44

Chapter 4: Save your fin, listen to your kin – promoting survival through kin recognition	49
4.1 Summary	49
4.2 Introduction	50
4.3 Methods	52
4.4 Results	56
4.5 Discussion	59
 Chapter 5: Age matters – embryos differentially respond to threat cues based on ontogenetic proximity	 64
5.1 Summary	64
5.2 Introduction	65
5.3 Methods	67
5.4 Results	71
5.5 Discussion	76
 Chapter 6: General Discussion	 79
 References	 90
Appendices	116
Appendix 1: Chapter 2 pilot trial	116
Appendix 2: Chapter 4 pilot trial	117

List of Tables

Table 1.1: Comparison of key life history characteristics between two common coral reef damselfish species.	13
Table 2.1: Effect of three parental predator treatments on embryonic offspring's responses to five olfactory cues in <i>Acanthochromis polyacanthus</i> .	26
Table 3.1: Experimental design for the embryo treatments and trials examining associative learning in embryos.	40
Table 3.2: Change in heart rate of embryonic <i>Amphiprion melanopus</i> in relation to the type of cue and the proximity to the time of hatching.	42
Table 3.3: Pilot study comparing the difference between changes in heart rate elicited by introducing a seawater control cue on consecutive days.	42
Table 3.4: Analysis of the learning capabilities of <i>A. melanopus</i> embryos after a single olfactory conditioning event.	44
Table 4.1: Comparison of embryo masses of <i>A. polyacanthus</i> , <i>A. melanopus</i> and <i>Chrysiptera cyanea</i> .	55
Table 4.2: Comparison of the reaction of embryos of two species (<i>A. melanopus</i> and <i>A. polyacanthus</i>) to alarm cues sourced from kin, non-kin conspecifics and heterospecifics.	58
Table 5.1: Change in embryo heart rates elicited by cues from donors of varying ontogenetic proximities.	71
Table 5.2: A two-factor MANOVA testing Cue and Clutch, and the interaction between them, where juvenile size (4 measurements) were the dependent variables.	74
Table A2.1: Test of the potential for using frozen predator odours as a proxy for fresh cues.	116
Table A4.1: Assessment of the validity of using frozen embryos to produce chemical alarm cues in test trials.	117

List of Figures

Figure 2.1: Mean baseline embryo heart rates, for each parental treatment, prior to the introduction of trial cues.	25
Figure 2.2: Effect of parental predator treatment on offspring response to five test cues representing differing levels of threat, in <i>Acanthochromis polyacanthus</i> embryos.	27
Figure 3.1: Detection of chemical alarm cues in <i>Amphiprion melanopus</i> embryos.	41
Figure 3.2: Associative learning of predatory threats by <i>Amphiprion melanopus</i> embryos.	43
Figure 4.1: <i>A. melanopus</i> embryo reactions to cues from kin and non-kin conspecifics, and heterospecifics.	57
Figure 4.2: <i>A. polyacanthus</i> embryo reactions to cues from kin and non-kin conspecifics, and heterospecifics.	58
Figure 5.1: Four morphological measurements (mm) taken from each three week old juvenile <i>A. polyacanthus</i> .	70
Figure 5.2: Reactions to cues of varying ontogenetic proximity in <i>Acanthochromis polyacanthus</i> embryos.	72
Figure 5.3: Mean juvenile size (mm \pm SE) across four morphological measurements: (a) standard length, (b) body depth, (c) peduncle depth, or (d) eye diameter; assessed on day 21 after hatching.	73
Figure 5.4a: Interaction plot between Cue and Clutch for juvenile standard length.	74
Figure 5.4b: Interaction plots between Cue and Clutch for juvenile size.	75
Figure 6.1: Chemical alarm cue relevancy spectrum.	84
Figure A4.1: Comparison of embryo reactions to alarm cues produced from either frozen or fresh embryos in both <i>A. melanopus</i> and <i>A. polyacanthus</i> .	118

Chapter 1: General Introduction

Prey animals are subject to predation from a range of species throughout their lives and the earliest life stages are often the most at risk from predators. The information that is relevant for predator avoidance and the survival of vulnerable juveniles can come from a variety of sources. These can include: innate knowledge, trans-generational parental knowledge of threats, the direct experience from surviving a strike, associative learning of a threat, and information from damage-released alarm cues from conspecifics and/or heterospecifics of varying ecological relevancies. To date, most of the research into the capacity of individuals to obtain information about predatory threats has focused on juveniles and adults. The present research focuses on the capacity of embryonic fishes to acquire information about predatory threats from their parents via non-genetic inheritance, and explores the use of embryonic olfaction to obtain information about risk from their environment prior to hatching.

Predation

Predation is one of the major drivers of population and community dynamics (Lima and Dill, 1990; Petorelli *et al.*, 2011). The behavioural and developmental mechanisms involved with avoiding predation often have great energetic costs (Houston *et al.*, 1993). As such, prey species have to balance the energy trade-off between antipredator behaviours and other fitness-promoting activities, such as foraging, growth and reproduction (Werner and Anholt, 1993; Brown and Smith, 1996). It is, therefore, important for prey to be able to recognise and distinguish between what actually poses a threat and what does not. Furthermore, predatory threats often vary greatly in space and time, and the relevance of specific predators will change with the ontogeny of the prey species, as its morphology, behaviour and habitat use changes (Sih *et al.*, 2000;

Wilbur, 1980). Thus, it is vital for species that will experience a wide range of predators throughout their lifespan to have a rapid and efficient means of identifying predators and updating the information they have on risk of predation. Research has shown that some species possess innate recognition of threats (Veen *et al.*, 2000; Hawkins *et al.*, 2004 and 2007; Chamaillé-Jammes *et al.*, 2013), while others upregulate this innate recognition or learn through association with visual, chemical and/or acoustic stimuli from predation events (Berejikian *et al.*, 2003; Epp and Gabor, 2008; Kindermann *et al.*, 2009; Mitchell *et al.*, 2011a).

Chemical alarm cues

Chemical alarm cues are chemicals involuntarily released when the upper epidermis of an aquatic animal is damaged, specifically, during a predation event (Smith, 1992). These damage-released odours alert nearby conspecifics and ecologically similar heterospecifics of a predatory threat in the area (Brown, 2003; Laforsch and Beccara, 2006). The prevalence and use of alarm cues has been demonstrated both in the laboratory and in the field, in a vast range of aquatic taxa (Chivers and Smith, 1998; Ferrari *et al.*, 2010a), including: fishes (Brown *et al.*, 2011a), amphibians (Waldman and Bishop, 2004), crustaceans (Hazlett, 1994), molluscs (Yamada *et al.*, 1998), echinoderms (Majer *et al.*, 2009) and insect larvae (Sullivan *et al.*, 2011). Recognition of such cues is an innate mechanism, and through associative learning individuals can couple the presence of this odour with the sight or smell of a predator and affirm the predator as a threat (Ferrari and Chivers, 2013; **Chapter 3**). Once an individual has learned a predatory risk, others can socially learn the threat by observing the reaction of closely related species to the predator (Griffin, 2004; Crane and Ferrari, 2013; Manassa *et al.*, 2013a). Moreover, some prey species are able to generalise learned predator identities to closely related predatory species that also likely pose a threat (Ferrari *et al.*, 2007; Webb *et al.*, 2010; Brown *et al.*, 2011b; Mitchell *et al.*, 2013). The use of alarm cues in determining risk has been repeatedly demonstrated to increase

antipredator behaviours, and more importantly, survival (Chivers and Smith, 1998; Ferrari *et al.*, 2010a; Brown *et al.*, 2011a).

Threat sensitive antipredator behaviour

A vast array of species are able to recognise both conspecific and heterospecific alarm cues; further, they can also differentiate between the cues based on the level of threat they signify. Research has shown that prey species can react in a graded manner to cues based on: cue concentration (Mirza and Chivers, 2003a; Marcus and Brown, 2004; Kesavaraju *et al.*, 2007; Hawkins *et al.*, 2007); the proximity of the life stage of the donor (cue source individual) (Mirza and Chivers, 2002; Sullivan *et al.*, 2003; Lönnstedt and McCormick, 2011); the phylogenetic proximity of the donor (Dalesman *et al.*, 2007; Mitchell *et al.*, 2012); and predator diet cues (Belden *et al.*, 2000; Hill and Weissburg, 2014). Presumably, by matching the level of antipredator response to the level of threat implied by a cue, prey are able to avoid unnecessary responses to non-predators and thus optimise their energy expenditure. Furthermore, the level of risk denoted by an olfactory cue can impact the magnitude of learning response through association (Mitchell and McCormick, 2013). Such refined and widespread methods for identifying risk and the appropriate responses demonstrate how important early and accurate recognition of threats are to predator avoidance. This is particularly pertinent in the early life stages of organisms, where predator-induced mortality rates are often highest (O'Donoghue, 1994; Qian and Chia, 1994; Almany and Webster, 2006). So, is it possible for prey to impart predatory knowledge to their offspring to give them a head start in life? This information could be vital for early life stage individuals, especially in highly diverse ecosystems, like coral reefs, which have numerous predatory threats.

Parental effects

Parental effects, involve the phenotypic (non-genetic) inheritance of traits. This transfer of traits can be a result of parental attributes, the way in which parental environment influences their phenotype, or a combination of the two, and parental effects can influence their offspring's phenotype, development and behaviour (Bernado, 1996; Green, 2008; Russell and Lummaa, 2009; Zimmerman *et al.*, 2016). Heritable attributes can include the size and body condition of the parent(s). In coral reef fishes, for example, larger females produced more eggs (Saenz-Agudelo *et al.*, 2015), and parents in better condition showed higher reproductive output and produced more successful offspring with increased survival (Donelson *et al.*, 2008). Aspects of the parental environment that can impact the resulting progeny and their survival include temperature (Green and McCormick, 2005; Páez *et al.*, 2009; Burgess and Marshall, 2011; Pajk *et al.*, 2012) and food availability (Kerrigan, 1997; McCormick, 2003; Kyneb and Toft, 2006; Warner *et al.*, 2015). Parental effects can occur via both parents, but maternal and paternal effects usually affect offspring in different ways (Hunt and Simmons, 2000; Wisenden *et al.*, 2011; Kroll *et al.*, 2013). For instance, by conducting a diallel cross breeding experiment, Green and McCormick (2005) found that paternal influences largely explained variation in larval growth rates, maternal traits influenced egg clutch traits, and the combination of maternal and paternal effects contributed to morphological and developmental differences between hatching and metamorphosis in a coral reef fish.

Offspring can benefit from their parents equipping them to survive in a specific environment (Bestion *et al.*, 2014) via the adaptive significance of parental effects. On the other hand, by gearing offspring to a specific future environment, it can mean the offspring are at a disadvantage should the environmental conditions they experience differ from their parents (Part III of Mousseau and Fox, 1998a; Beckerman *et al.*, 2006; Marshall and Uller, 2007). Coslovsky and Richner (2012) found that there were

developmental costs associated with offspring being geared to suit an environment that had changed. Offspring produced by parents in a high predator environment, but raised in a predator-free environment, fledged later than those raised in an environment that matched the parental environment.

Predator-induced parental effects and their adaptive significance

Parental effects can also be influenced and/or induced by the presence of predators, impacting the development and behaviour of offspring. For instance, mother lizards exposed to predators produced heavier offspring with longer tails (Shine and Downes, 1999), larger offspring that grow faster and have increased reproductive success in rats (Besson *et al.*, 2014), and smaller offspring with larger wings were found in birds (Coslovsky and Richner, 2011a). Parents in high risk environments can also produce offspring that display higher levels of antipredator behaviours; previously observed in insects (Storm and Lima, 2010) and fishes (Giesing *et al.*, 2011). All of these developmental, physiological and/or behavioural traits, which have been passed to the progeny, have been linked to more effective predator avoidance and increased offspring survival. However, research has also demonstrated that survival costs can be incurred by offspring of predator-exposed parents. These include: increased parasite loading in birds (Coslovsky and Richner, 2011b); decreased antipredator behaviours and survival in sticklebacks (McGhee *et al.*, 2012); and reduced associative learning capabilities in fishes (Eaton *et al.*, 2015; Feng *et al.*, 2015). It is, however, possible for offspring to compensate for any potential maladaptive consequences of predator induced parental effects by demonstrating plasticity in development and/or behaviour in response to their own environment and experiences (Beckerman *et al.*, 2006; Stratmann and Taborsky, 2014; Feng *et al.*, 2015).

A number of non-genetic mechanisms have been proposed for the way in which information on predatory risk from adults can be transferred to offspring. For instance, environments with higher levels of predatory threats increase stress levels in prey species, which in turn, affects the hormonal composition of parents (i.e., cortisol and other stress hormone levels increase) (Monclus *et al.*, 2009). These stress hormones can be transferred into offspring during oogenesis and impact any resultant offspring developing at that time, which were noted in fishes (Gagliano and McCormick, 2009; Sopinka *et al.*, 2014) and birds (Rubolini *et al.*, 2005; Coslovsky *et al.*, 2012). Moreover, Sheriff *et al.* (2015) found that the greater the predatory threat, and thus level of maternal stress, the more prolific the effects on resultant offspring; as such, reduced reproductive success was seen across multiple subsequent generations. Another potential mechanism for predator induced parental effects is epigenetic transfer, in that parental experience affects the way in which offspring's genes are expressed, altering phenotypic traits (Youngson and Whitelaw, 2008; Formanek *et al.*, 2009). Mommer and Bell (2014) found predator exposed stickleback mothers produced larger embryos which developed at a faster rate than offspring from control mothers. Genetic analysis showed predator-exposed and control mothers down-regulated and up-regulated different genes, which were inherited by resultant offspring and induced the observed phenotypic and developmental differences.

Surprisingly, no research into predator-induced parental effects has been carried out on a marine fish species. Nor has research demonstrated whether any species is able to transfer information regarding a specific predatory threat to their offspring via parental effects (**Chapter 2**). In environments with multiple and constantly changing predatory threats, such as coral reefs, knowing what specifically poses a threat can prevent an unnecessary response to low risk cues and/or predators, and increase the probability of survival.

Embryonic awareness and learning

Predator-induced mortality rates are often highest in early life stages (Wilbur, 1980; Webster, 2002; Almany and Webster, 2006). While, the prevalence of alarm cue and/or predator cataloguing has been widely studied for juvenile stage fishes, there have been no studies conducted on the cognitive development of embryos within marine systems. However, research on amphibians has demonstrated that embryos who experience higher concentrations of alarm cues elicit greater antipredator behaviours as juveniles, suggesting that threat-sensitive awareness exists in embryos (Ferrari and Chivers, 2009a; Ferrari and Chivers, 2010; Ferrari *et al.*, 2010b). Additionally, Oulton *et al.* (2013) found that rainbowfish embryos have innate recognition of a native predator odour, with odours that signify a greater predatory threat inducing a greater increase in heart rate than an odour from a lesser threat. In the context of this thesis, the term 'innate recognition' will refer to a reaction observed in an embryo in response to an olfactory stimulus, which the embryo itself has not previously experienced.

Learning has also been demonstrated in embryonic stage organisms in a variety of taxa, including multiple amphibious species (Hepper and Waldman, 1992), cichlid fish (Nelson *et al.*, 2013), cephalopods (Romagny *et al.*, 2012), and mites (Peralta-Quesada and Schausberger, 2012). Furthermore, while no studies have been conducted on embryonic fishes, evidence suggests that the relevance and concentration of the damage-released odour, coupled with a predator odour, can impact the extent to which an individual labels an odour as a threat. For instance, conditioning with higher concentrations of alarm cues, or those emitted from donors of closer ontogenetic proximity, elicit stronger antipredator responses to the learned predator odour (Ferrari and Chivers, 2009a; Ferrari and Chivers, 2010; Mitchell and McCormick, 2013). Early recognition and learning of threats could allow for informed selection of habitats with fewer predatory risks upon settlement (Johnson and Strathmann, 1989; Wennhage and Gibson, 1998; Vail and McCormick, 2011; Dixon,

2012). Despite the combined supporting evidence for both learning in embryos and the use of threat cues to avoid more risky habitats on settlement, the capacity for marine fish embryos to associatively learn predatory species is yet to be investigated (**Chapter 3**).

Advantages of early recognition of threats

Early detection of risks can promote numerous survival advantages for developing embryos. For instance, elasmobranch embryos (bamboo sharks, *Chiloscyllium punctatum*, and thornback rays, *Raja clavata*) have demonstrated an innate ability to recognise predatory threats using electroreception and temporarily suspend gill movements, which reduces their chance of being detected (Kempster *et al.*, 2013; Ball *et al.*, 2015). This can be an important antipredation survival mechanism for embryos of egg laying species of sharks and rays, as offspring do not receive parental care after spawning (Reynolds *et al.*, 2002). Identification of threats in the immediate environment can also lead to premature or delayed hatching, potentially allowing prey to escape predation. This phenomenon has been identified in a number of amphibious species, whereby vibrational (Warkentin, 2000 and 2005) and/or chemosensory cues (Sih and Moore, 1993; Chivers *et al.*, 2001; Johnson *et al.*, 2003; Mandrillon and Saglio, 2007) have modified hatching times allowing prey to evade capture. The ability of embryos to adjust their hatching time seems to be dependent on the type of predator present in their environment. For example, Ireland *et al.* (2007) found that egg predators caused premature hatching at a smaller size and the presence of a larval predator delayed hatching; but, the presence of both predators caused no detectable change in hatching time or development, in comparison to controls.

Being exposed to predator and/or alarm cues during embryogenesis can prompt alterations in the development of morphological traits and/or induced defences in an individual, which can increase their chance of survival. Predator-induced defences are

phenotypic traits that differ from the 'typical morph' of a species and their development is initiated by chemical cues; for instance, the formation of spines, or protective helmets in *Daphnia*. *Daphnia* with these induced defences are at a reduced risk from predation, as it means they often exceed the maximum size of prey consumed by their normal predators (Lass and Spaak, 2003; Van Donk *et al.*, 2011; Gilbert, 2013). Additionally, in predator exposed embryonic amphibians, larvae hatched with shorter bodies and deeper tails, making them more proficient swimmers (Laurila *et al.*, 2001). Moreover, these amphibians adopted a variety of different hatchling morphologies depending on the type of predator experienced as an embryo (Laurila *et al.*, 2002). Exposure to predator and/or chemical alarm cues during embryogenesis can also speed up development in zebrafishes (Mourabit *et al.*, 2010) and the common frog (Segev *et al.*, 2015). This allows the individual to 'fast track', through the vulnerable, immobile embryonic stage, to hatching, where the juveniles will be able to actively avoid predators on detection of threat cues.

Threats detected and learned before hatching can alter behavioural characteristics of larvae and juveniles after hatching. Cuttlefish showed a bias towards turning left if they had experienced predator cues throughout embryogenesis (Jozet-Alves and Hébert, 2013). Individuals with enhanced lateralisation (having a stronger preference for right or left) have shown improved abilities of simultaneously foraging for food whilst remaining alert to predatory threats (Rogers *et al.*, 2004; Dadda and Bisazza, 2006). Similarly, amphibians and freshwater fishes that have experienced or learned predatory threats in the embryonic stage display a higher propensity for antipredator behaviours as juveniles (Mathis *et al.*, 2008; Nelson *et al.*, 2013; Dalesman *et al.*, 2015). Gazzola *et al.* (2015) investigated the effects of embryonic predator exposure in the agile frog (*Rana dalmatina*), by measuring morphological, behavioural and neurophysiological traits for over a month after hatching. In accordance with the findings of other studies, predator treated embryos hatched later, were smaller at hatching and displayed greater

antipredator behaviours (i.e., reduced overall activity) initially, but the magnitude of the antipredator response decreased with increasing age. Interestingly, the neurophysiological reactions to predator cues were greater in the predator treated individuals, but unlike the antipredator behaviours, the neurophysiological response to the same threat odour did not diminish with age. This suggests that early predator exposure 'hard-wired' an increased fear response in this species, but with age and experience, they were able to adjust the magnitude of the associated antipredator behaviour to match the level of threat inferred by the cue. Hence, while early experience of threats can provide a baseline for traits that can increase the survival potential for juveniles post-hatching, it is important to update and refine these traits to ensure they correspond to the current environmental conditions, in accordance with the threat-sensitive hypothesis (Helfman, 1989),

Disadvantages of embryonic predator exposure

Exposure to predatory threats during embryogenesis can also have negative consequences for post-hatching juveniles. For instance, embryos of great crested newts (*Triturus cristatus*), which developed in the presence of predators had higher mortality rates than controls, but time of hatching and morphology did not differ between treatments (Jarvis, 2010). This implies that the non-consumptive, likely stress inducing, effects of predator presence during embryo development can also carry survival costs for prey species. This could be partly due to the fact that chemical cues denoting risk can induce increases in heart rate (tachycardia) in embryos (Oulton *et al.*, 2013), which carries metabolic and immune function costs (Slos *et al.*, 2009). Furthermore, although induced/premature hatching allows prey to escape an immediate predatory threat, once hatched, the larvae are often less developed than they would normally be (Warkentin, 1995). Consequently, they are more vulnerable to predation than their larger, more developed counterparts (Petranka *et al.*, 1987; Sih and Moore, 1993), due to gape limitations of predators (Holmes and McCormick,

2010a). Hettyey *et al.* (2011) conducted a comprehensive study on the survival implications of induced defences in response to various levels of predatory threats in the agile frog (*Rana dalmatina*). They found higher mortality rates in tadpoles which manifested the strongest morphological changes in response to high predatory threat exposure during development. Concurrently, predator-induced life history changes have been shown to reduce the reproductive output and success of some taxa; i.e., aphids (Dixon and Agarwala, 1999) and *Daphnia* (Hammill *et al.*, 2008). However, in general, the costs incurred by the manifestation of predator-induced traits tend to be quite low and only some research has successfully identified direct and/or delayed costs linked to this type of phenotypic plasticity (Scheiner and Berrigan, 1998; Van Buskirk and Saxer, 2001)

Gaps in the literature

While research into the olfactory capabilities of embryos is increasing, there are still many gaps in our current knowledge of the field. Specifically, previous studies into threat sensitive responses have focused on alarm cue concentration, and none have identified whether embryos can distinguish between alarm cues from different sources of varying ontogenetic and phylogenetic proximities (**Chapters 4 and 5**). As previously outlined, research into juvenile and adult aquatic species has demonstrated the importance of being able to differentiate between cues based on their relevance. Discriminating between cues and responding in a graded manner can increase the efficiency of antipredator behaviours while conserving energy for other fitness related activities. This can increase an individual's chance of survival, so it would be ecologically beneficial for them to be able to discriminate between cues from the earliest possible age.

Despite the growing research interest in embryonic environmental awareness and learning capabilities, the capacity for coral reef fish embryos to detect and/or react to

threat cues has never been investigated (**Chapters 3 – 5**). To date, studies have shown that reef fish embryos can obtain information using olfactory cues, by identifying their propensity to imprint on natal odours (e.g., host anemones or reefs), which biases their selection of settlement sites (Arvedlund and Nielsen, 1996; Arvedlund *et al.*, 1999 and 2000; Atema *et al.*, 2002; Gerlach *et al.*, 2007a; Dixon *et al.*, 2008; Dixon, 2012). Hence, olfactory cues play a seemingly important role in early life stages of coral reef fish, but the ability of embryos to detect and react to odours is yet to be investigated.

Relevance of coral reefs and study species

Coral reefs are one of the most biodiverse ecosystems in the world (Bellwood and Hughes, 2001). As such, prey reef fish species are faced with a diverse range of predatory threats with a suite of different foraging tactics (Heinlein *et al.*, 2010). It was estimated that on average 56% of juvenile reef fishes are consumed within 1-2 days of settlement (Almany and Webster, 2006), and embryos of demersal reef fish species experience high predation rates, despite often being aggressively defended by their parents (Emslie and Jones, 2001). Thus, in order to survive, coral reef fishes need to be adept at continually and rapidly learning new threats and updating their innate knowledge; as well as cataloguing alarm cues and predator odours based on the level of risk they denote.

The use of chemical alarm cues in determining risk and learning predators has been identified in a range of damselfishes (Pomacentridae) (Holmes and McCormick, 2010b; Lönnstedt *et al.*, 2012a; Mitchell *et al.*, 2012). For my research, I selected two common coral reef damselfishes as study species (*Acanthochromis polyacanthus* and *Amphiprion melanopus*), based on their ability to breed successfully in captivity, and further, they possess very different life history characteristics (Table 1.2). Studying these two species allowed me to not only determine the olfactory capabilities of coral reef fish embryos, but make a comparison between species with and without a pelagic

larval phase, to assess if there is a difference in embryonic risk assessment depending on life history characteristics, or if a general trend exists across demersally spawning species.

Table 1.2: Comparison of key life history features of two common species of coral reef damselfish, *Acanthochromis polyacanthus* and *Amphiprion melanopus* (Doherty *et al.*, 1995; Green and McCormick, 1999; Kavanagh, 2000; Green, 2004).

Life history trait	<i>Acanthochromis polyacanthus</i>	<i>Amphiprion melanopus</i>
Mean egg length (mm)	4.0	2.2
Eggs per clutch	250 – 550	300 – 1000
Mean egg duration (d)	11	7.5
Approximate hatch time	Midday	Dusk
Mean length at hatching (mm)	5.8	2.5
Larval duration (d)	0	15 – 22
Parental care period	≤ 3 months post-hatching	Embryogenesis only

Self-recruitment and kin recognition

Acanthochromis polyacanthus have limited dispersal (Miller-Sims *et al.*, 2008), even after being brooded by their parents for up to three months (Kavanagh, 2000). Consequently, *A. polyacanthus* have very distinct genetic populations across the Great Barrier Reef (Doherty *et al.*, 1994; Planes *et al.*, 2001). However, despite having a pelagic larval stage, many other coral reef fish species also demonstrate high levels of recruitment back to their natal reefs at settlement (Berumen *et al.*, 2012; Jones, 2015). For instance, some coral reef fish species' self-recruitment levels have been shown to be: over 30% in the *Amphiprion polymnus* (Jones *et al.*, 2005); up to 60% in *Pomacentrus amboinensis* (Jones *et al.*, 1999); 75% in *Pomacentrus coelestis* (Patterson *et al.*, 2005); 60% in *Amphiprion percula* and 52-72% in *Chaetodon*

vagabundus (Almany *et al.*, 2007); and up to 64% in *Coris picta* (Patterson and Swearer, 2007). The higher the likelihood of encountering one's genetic relatives (e.g., parents and/or siblings), the greater the probability of kin recognition occurring within that population and/or species (Carreno *et al.*, 1996; Arnold, 2000). Kin recognition, the ability for an individual to identify their relatives amongst other conspecifics, is a widespread phenomenon identified across numerous taxa, including amphibians (Blaustein and Waldman, 1992), birds (Krause *et al.*, 2012), fish (Frommen *et al.*, 2013), insects (Whitehorn *et al.*, 2009), mammals (Mateo, 2003) and reptiles (Léna and Fraipont, 1998). The high self-recruitment levels in both *A. polyacanthus* and *A. melanopus* mean they provide excellent model species to study kin recognition in embryos (**Chapter 4**).

One of the proposed benefits of recognising kin is improved predator avoidance and antipredator behaviours (FitzGerald and Morrisette, 1992; Brown, 2002). For instance, the release of chemical alarm cues has, at times, been considered to be an altruistic act, but if relatives are nearby during a predation event, their kin will also benefit from the alarm cue's release (Waldman, 1982). Furthermore, Griffiths *et al.* (2004) showed that when brown trout associated with familiar individuals, they had increased foraging rates and responded more quickly to predatory threats. Despite there being a lot of literature on kin recognition and its benefits, no research has determined whether embryos are able to differentiate kin from non-kin. Furthermore, kin selection is a much understudied field of research in the marine environment (Kamel and Grosberg, 2013), which is, in part, due to a widely held assumption that it would be hard for siblings to remain together during their dispersive larval phase. Yet, a growing amount of research has demonstrated high levels of sibling and kin association in settled marine organisms; i.e., invertebrates (Grosberg and Quinn, 1986; Amar *et al.*, 2007; Amar *et al.*, 2008) teleost fishes (Selkoe *et al.*, 2006), and more specifically, some species of coral reef fishes (Planes *et al.*, 2002; Buston *et al.*, 2009; Bernardi *et al.*, 2012; Selwyn

et al., 2016). Despite this, research is yet to provide empirical evidence for coral reef fishes being able to distinguish between kin and non-related conspecifics.

Aims and thesis outline

This thesis examines the impact of parental and embryonic predator environments on offspring of coral reef fishes, using laboratory-based experiments. In **Chapter 2**, I determine whether breeding pairs of a common coral reef damselfish, *Acanthochromis polyacanthus*, are able to convey information regarding local and relevant predatory threats to their offspring through non-genetic, parental effects. In the following three chapters (**Chapters 3 – 5**), I explore the olfactory capabilities of the embryonic stages of two different species of coral reef damselfish, namely *A. polyacanthus* and *Amphiprion melanopus*. In particular, I evaluate the capacity for embryonic fishes to associatively learn from, and differentiate between, chemical alarm cues. **Chapter 3** determines whether embryonic clownfishes are able to detect damage-released chemical alarm cues before they hatch, and if they can use this innate recognition to learn specific predatory threats. **Chapter 4** then establishes whether embryos of both *A. melanopus* and *A. polyacanthus* are able to differentiate between chemical alarm cues from donors of varying levels of relatedness. Specifically, I will evaluate if the embryos react differently to cues derived from kin, or other conspecific cues, or heterospecific cues. Lastly, **Chapter 5** explores whether embryos can also distinguish between chemical alarm cues from conspecific individuals based on their ontogenetic proximity and relevancy and if this early exposure affects juvenile size post-hatching. This thesis provides the first insights into the effects of embryonic and parental predator presence on offspring, contributing to a more complete understanding of how predator-prey relationships can affect early life stages, habitat selection and recruitment in coral reef fish ecosystems.

Chapter 2: Parents know best – conveying predator information to offspring through parental effects

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2.1 Summary

In highly biodiverse systems, such as coral reefs, prey species are faced with predatory threats from numerous species. Recognition of predators can be innate, or learned, and can help increase the chance of survival. Research suggests that parents may be able to convey predator information to offspring, providing them with an adaptive advantage. Breeding pairs of a damselfish (*Acanthochromis polyacanthus*) were subjected to one of three olfactory and visual treatments (predator, herbivore, or control), and their developing embryos were subsequently exposed to five different chemical cues. Analysis of embryonic heart rates showed that predator-treated parents passed down relevant threat information to their offspring, through parental effects. This is the first time that transgenerational recognition of a specific predator has been found for any organism. This phenomenon could influence predator-induced mortality rates and enable populations to adaptively respond to fluctuations in predator composition and environmental changes.

2.2 Introduction

Predation is a major driving force in population and community dynamics (Pettorelli *et al.*, 2011). Antipredator behaviours are often energetically costly and detract from fitness-promoting activities, like foraging for food (Werner and Anholt, 1993). Furthermore, the types of predators that pose threats to individuals also change with habitat and life history stage (Lönnerstedt and McCormick, 2011). In order to increase their fitness and chance of survival, individuals need to be able to recognise predatory threats, and react in a manner that matches the level of risk experienced (Helfman, 1989). Some species have an innate recognition of predators (Hawkins *et al.*, 2004), while for others, learning plays an important role in the identification of relevant threats (Crane and Ferrari, 2013). This learning tends to occur through conditioning events, which in aquatic taxa can be achieved using chemical alarm cues (CAC). These olfactory cues are released when the epidermis of an aquatic organism is damaged, alerting both conspecifics, and cognisant heterospecifics, of nearby predatory risks. The coupling of ecologically relevant CAC with predator odours, which are passively released, can allow prey to identify the predator as a threat through this common form of Pavlovian conditioning (Ferrari *et al.*, 2010a). Some species use this olfactory associative learning to further refine innate predator recognition (Berejikian *et al.*, 2003). This learned predator information can then be passed on to other individuals within a guild through social learning (Manassa *et al.*, 2013a). Once learned, identities of predators can also be generalised to cues from closely related species that may pose a threat (Ferrari and Chivers, 2009a). Despite the potential advantages of parents passing on information to their offspring about the identity of relevant predators, the extent to which this occurs is unknown.

Parental effects involve the non-genetic inheritance of maternal and/or paternal traits. This transfer can occur either directly, through inheritance of non-genetic material from one or both parents (i.e., during gametogenesis), or indirectly, as a result of parental

behaviour and the care provided to offspring (i.e., during embryogenesis; Bernado, 1996; Heath and Blouw, 1998). Parental environment and/or attributes can influence the phenotypic outcome, in terms of growth and development, and behaviour, of any offspring produced (Green, 2008; Bennett and Murray, 2014). Research has shown that both male and female parents can contribute to this phenotypic inheritance, with paternal and maternal attributes affecting offspring in different ways (Hunt and Simmons, 2000; Green and McCormick, 2005; Wisenden *et al.*, 2011; Kroll *et al.*, 2013). In addition, environmental cues can be transferred to offspring while they are still developing inside their mother, which can consequently alter offspring phenotypes (Ledón-Rettig *et al.*, 2013); though we are not aware of evidence that this phenomenon occurs in fishes.

Predator presence can also impact offspring through parental effects. Experimentally exposing breeding pairs to predators can cause changes in antipredator behaviour of offspring (Storm and Lima, 2010; McGhee *et al.*, 2012) as well as their growth and development (Shine and Downes, 1999; Agrawal *et al.*, 1999; Coslovsky and Richner, 2011a) in a range of taxa. Several potential pathways through which this transfer occurs have been suggested. For example, predator presence can induce stress, which in turn can alter the hormonal composition of gametes during development, resulting in changes in offspring quality (McCormick, 1998; Coslovsky *et al.*, 2012). Other research suggests epigenetic mechanisms of transfer, whereby changes in gene expression generate phenotypic differences in offspring (Youngson and Whitelaw, 2008; Mommer and Bell, 2014). Such predator-induced parental effects have been shown to carry-over across all subsequent life stages of the offspring, even into adulthood (Roche *et al.*, 2012). This phenotypic inheritance can even be detected across multiple generations, affecting factors such as maturation rates and reproductive success (Mondor *et al.*, 2005; Sheriff *et al.*, 2010; Auld and Houser, 2015; Walsh *et al.*, 2015). The prevalence of predator-induced parental effects across a wide

range of taxa and environments suggests they could confer an adaptive advantage (Mousseau and Fox, 1998b; Marshall and Uller, 2007; Bestion *et al.*, 2014). However, there are also examples of maladaptive consequences of parental effects in stressful environments, such as increased parasite loading (Coslovsky and Richner, 2011b), decreased antipredator behaviours (McGhee *et al.*, 2012), and metabolic and functional disorders (Schuler and Orrock, 2012) in offspring.

Predator-induced mortality rates in juveniles are exceptionally high in coral reef fishes (Almany and Webster, 2006), as they are for many organisms with complex life cycles (Wibur, 1980). Recent research has shown that embryonic anemonefish (*Amphiprion melanopus*) are able to learn predatory threats, through ambient odours, prior to hatching (Atherton and McCormick, 2015). Many coral reef fishes recruit to their natal reefs (Berumen *et al.*, 2012), and identification of known predator odours can influence habitat selection when settling on the reef (Dixon, 2012). Therefore, one would expect that the more parents can aid their offspring with recognising cues that are relevant to the environment they are likely to settle in, the higher their chance of survival. Hence, if parents can impart the ability to identify predators relevant to their natal habitat to their offspring, they are more likely to be able to avoid these at settlement (Vail and McCormick, 2011). Surprisingly, it has not yet been identified whether specific predator information can be transmitted via parental effects.

Consequently, the aim of my research was to determine if transgenerational predator recognition occurs in a common damselfish on Indo-Pacific reefs, the spiny chromis, *Acanthochromis polyacanthus*. I achieved this by subjecting breeding pairs to one of three olfactory and visual treatments (predator, herbivore, or control), and any offspring produced were tested for their reactions to one of five chemical cues (parental predator, novel predator, herbivore, embryo chemical alarm cue, or seawater). Embryo alarm cues were used in the trials to provide a baseline for how embryos respond to a high risk cue, and a novel predator odour was used so I could determine if any

reactions to the parental predator odour were embryos responding to a threat cue in general, or to the transgenerational relay of specific predator information. Here, I show that not only are parents able to convey specific predator information to their offspring, but embryonic damselfish can also innately differentiate between chemical cues and react according to their level of threat.

2.3 Methods

a) Study species

Acanthochromis polyacanthus (Pomacentridae) is an ideal model study species for research into parental effects in coral reef fishes because they can be bred and reared in captivity, which is partly due to *A. polyacanthus* lacking a pelagic larval stage. Embryogenesis varies in duration from 8 – 14 days, and the parents care for their offspring for up to three months post-hatching, prior to juvenile migration (Kavanagh, 2000).

The model predator species used in treatments and test trials was the coral trout (*Plectropomus leopardus*, Serranidae), a known, sympatric predator of adult and juvenile *A. polyacanthus* (St. John, 1999). The dottyback (*Pseudochromis fuscus*, Pseudochromidae) was used solely as a cue in embryo trials and represented the ‘novel predator’ in this experiment. *P. fuscus* is phylogenetically distant from *P. leopardus*, but is another sympatric piscivore of both embryonic and juvenile stage damselfishes (Emslie and Jones, 2001; Feeney *et al.*, 2012). The herbivorous barred rabbitfish (*Siganus doliatus*, Siganidae) was also used in both parental treatments and test trials, and represented a low risk stimulus as a non-threatening coral reef fish species.

b) Animal husbandry

All research was completed at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University, Townsville, Australia. Twenty-one adult breeding

pairs of *A. polyacanthus* were kept in an outdoor, isolated system, with each pair in a 70 L tank. The system contained seawater maintained at $28 \pm 1^\circ\text{C}$ and a salinity of 35 ppm, with a normal light:dark (12.5:11.5 h) diurnal cycle during the summer months. Each tank was well-aerated and contained half of a terracotta pot, and each pair was fed pelleted food twice daily. Each tank was checked daily for egg clutches, and if found, clutches were left with their parents during embryogenesis. At 9 days after fertilisation, all eggs in the clutch were collected by carefully cutting the tissue (holdfast) adhering the eggs to the terracotta pot, and were transferred into a 1 L beaker. The beaker contained water from the parental tank, was well-aerated, and kept in a water-bath in the experimental laboratory to maintain the temperature at that of the parental tank.

c) Parental treatments

Each breeding pair was randomly assigned to one of the three treatments (predator, herbivore or control), which involved multiple conditioning events, carried out in the morning on a weekly basis, until all test trials had been completed. If egg clutches were present in any parental tanks, treatments were not undertaken for those specific pairs to avoid direct embryo exposure to parental treatment cues. Out of the 21 pairs set up, 3 pairs from each treatment successfully reproduced in each treatment. In order to prevent cross contamination of cues, the water flow was shut off to each tank, and 8 L of the water in each treatment tank was removed, just prior to treatments, to allow room for the cues to be added without the risk of overflow into the recirculated system. The predator treatments involved careful introduction of a fibre glass model of *P. leopardus* (40 cm SL) and 4 L of predator odour, which was slowly added using a funnel tube, so as to reduce the level of human disturbance imposed on experimental fishes. Once per month (every four treatments), to reinforce that the predator odour represented a threat, the 4 L of predator odour was paired with 50 ml of adult *A. polyacanthus* chemical alarm cue (CAC). During the other three out of four predator treatments, 50

ml of seawater (instead of CAC) was introduced with the predator cue, to ensure consistency of disturbance between treatments. The herbivore treatment involved introducing a fibre glass model of *S. doliatus*, combined with 4 L of herbivore odour and 50 ml of seawater. To act as a disturbance control, a plastic container, 4 L of seawater and 50 ml of seawater were introduced to breeding pairs allocated to control tanks. After 15 min of cue exposure, around 60% of the water in each tank was siphoned out and the tank was refilled to just below the level of the outflow pipe. The water was left to allow diffusion of all treatment cues for around 4 hours before water flow was resumed and resulting water dumped out of the isolated system. This procedure ensured that there was no cross-contamination of any cues among treatment tanks.

d) Stimulus preparation

(i) Treatment odours

Seawater used in the control treatment was obtained from the outdoor parental system to ensure that there was no contamination from other fish odours and that water quality parameters were kept constant. The predator odour was obtained from one of three (individual used was changed each week) adult coral trout, *Plectropomus leopardus* (40 cm SL), which were not fed 24 h prior to cue collection to minimise the amount of dietary cues in the water. The predator was kept overnight in 70 L of seawater, and the odour-infused water was then used to treat the predator-assigned breeding pairs. The herbivore odour was produced using the same method, but with the barred rabbitfish, *Siganus doliatus*, (20 cm SL) as the cue donor, and the holding tank contained 35 L of seawater. Once a month, the predator odour was combined with an adult *A. polyacanthus* chemical alarm cue, to ensure the breeding pairs were identifying the coral trout as a threat. This alarm cue was created by making ten superficial cuts along each side of an adult (>7 cm SL) *A. polyacanthus*, that had been euthanised by a quick blow to the head, rinsing each side with 50 ml of seawater, using a coarse filter (0.75

mm pore size) to remove any particulate matter. One adult fish was used to make 100 ml of alarm cue, which was enough for two replicate treatments.

(ii) Trial odours

Embryo reactions were tested, using one of five different cues, which included: seawater control (SW), embryo chemical alarm cue (CAC), 'known' predator (coral trout, *P. leopardus*), novel predator (dottyback, *P. fuscus*), or herbivore (*S. doliatus*). The *A. polyacanthus* embryo CAC was made by crushing five embryos in a petri dish and mixing this with 5 ml of seawater. The resultant solution was filtered through filter paper to remove particulate matter, leaving the cue infused seawater; 1 ml of this cue was used in each test trial. Odours for the parentally taught predator and herbivore trials were collected from the water produced for the weekly parental treatments and 2 ml aliquots were placed in liquid nitrogen for a maximum of 2 weeks, to be defrosted and used when trials were carried out (see Appendix 1: Chapter 2 pilot trial). The dottyback (10 cm SL) was placed in 9 L seawater overnight to create the novel predator odour, and the resultant cue was again frozen in 2 ml aliquots. Again, the dottyback was starved 24 h prior to cue collection to minimise the presence of digested alarm cues in the test trial cues.

e) Embryo test trials

A total of 75 embryos were tested from each clutch produced; 15 embryos for each of the five cues. A single embryo was placed in 10 ml of seawater, sourced from the same temperature controlled system, under a dissecting microscope with a cold light, and allowed 2 min to acclimatise. The reaction elicited by the introduction of a cue was calculated by visually recording the embryo's heart rate for 30 s, carefully injecting 1 ml of cue into the seawater, and then recording the heart rate for a further 30 s. The change in the number of heart beats per 30 s, induced by the introduced chemical stimulus was then calculated. This procedure was repeated using four separate clutches from three breeding pairs (two pairs produced one clutch each and one pair

produced two clutches), for each parental treatment. Heart rate was used as the measured behavioural proxy because it is easily visible using a dissecting microscope and research has shown that predator presence induces not just behavioural changes, but also concurrent changes in heart rate (Höjesjö *et al.*, 1999).

f) Statistical analyses

(i) Baseline heart rates

A nested ANOVA model was conducted to assess if the actual, baseline embryo heart rates, prior to the introduction of trial cues, differed across parental treatments. This two-factor model tested parental treatment (fixed) and clutch (random and nested in parental treatment). Residual analysis showed that the raw initial heart rate data met the assumptions of ANOVA.

(ii) Changes in heart rates

A three-factor ANOVA was undertaken to determine whether the change in embryo heart rate was affected by: parental treatment (fixed: predator, herbivore, seawater); trial cue (fixed: parental predator, novel predator, herbivore, embryo alarm cue, seawater); and clutch (nested and random: 4 clutches per treatment). Tukey's HSD post-hoc tests were used to determine the nature of any significant differences found by ANOVA. The raw change in heart rate data also met the assumptions of ANOVA.

g) Ethical note

All procedures were approved by the James Cook University Animal Ethics Committee under the permit A1871.

2.4 Results

a) Baseline heart rates

Baseline embryo heart rates (beats per 30 s prior to cue introduction) did not differ significantly among parental treatments ($F_{2,891} = 2.269$, $P = 0.184$, Figure 2.1). The total

effect of clutch nested in parental treatment was significant and explained the variance in the ANOVA model ($F_{6,891} = 47.066$, $P < 0.0001$).

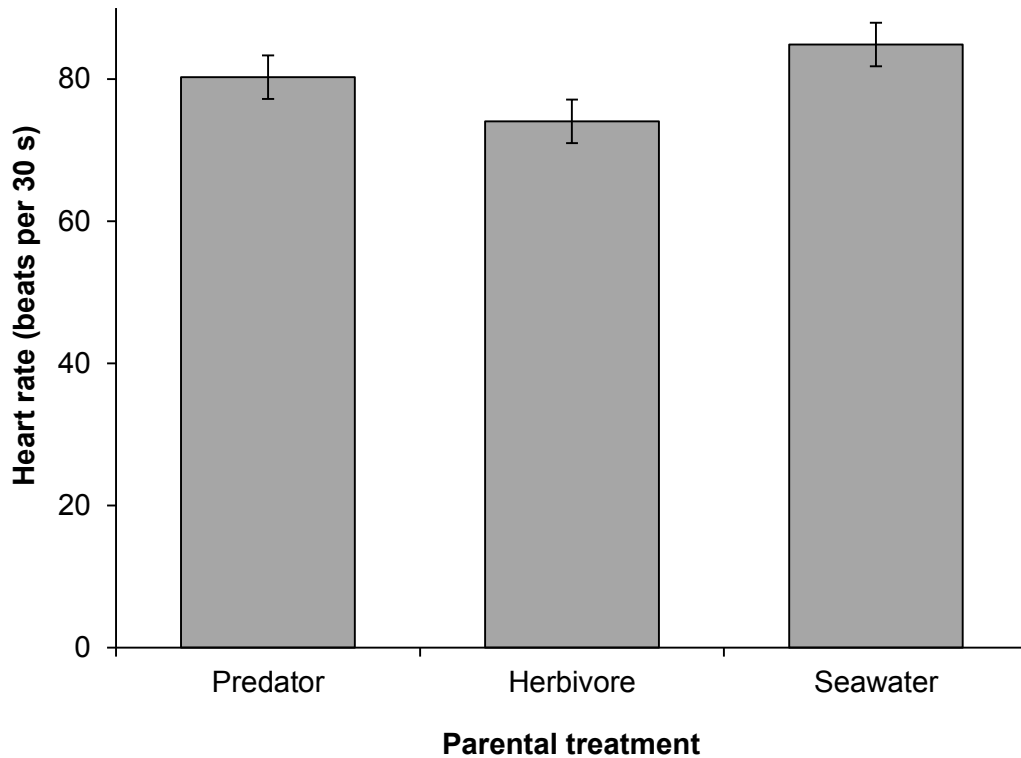


Figure 2.1: Mean baseline embryo heart rates for each parental treatment prior to the introduction of trial cues ($N = 300$ per column). Error bars represent the Tukey's 95% confidence limits.

b) Reactions to trial cues based on parental treatment

There was a significant interaction between parental treatment and embryo trial cue (Table 2.1; Figure 2.2). Following introduction of the parental predator odour, offspring of the predator treated parents showed an increase in heart rate (+10.13%) that was almost twice that of the herbivore and seawater control treated parents (+5.14%, +5.49%, respectively; Tukey's HSD: $P < 0.001$ for both comparisons; Figure 2.2). This contrasts with the heart rate changes induced by the seawater, embryo alarm cue, novel predator and herbivore trial odours that did not differ among the three parental treatments (Tukey's HSD: $P = 1.00$ for each of the four aforementioned cues, when comparing across parental treatments; Figure 2.2). The clutch term nested in parental

treatment was also significant; i.e., there was a lot of variability in the embryonic responses between clutches produced by parental pairs as well as between the treatments to which they were exposed (Table 2.1).

Table 2.1: Comparison of the mean changes in heart rates of embryonic *Acanthochromis polyacanthus*, that were exposed to one of five cues (parental predator, novel predator, herbivore, embryo alarm cue, seawater), and whose parents had been exposed to one of three threat treatments (predator, herbivore, seawater) of four egg clutches.

Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Parental Treatment (P)	2	117.209	5.799	0.0353
Embryo Cue (E)	4	2620.842	615.207	<0.0001
P x E	8	90.900	21.338	<0.0001
Clutch (Parental Treatment) C(P)	6	20.102	5.766	0.0008
E x C(P)	24	3.487	0.153	1.0000
Residual	855	22.825		

c) Reactions to trial cues irrespective of parental treatment

On introduction of an embryo chemical alarm cue (CAC), embryos from all three parental treatments responded with a similar increase in heart rate (mean = +10.31%), which differed significantly from the mean increases in heart rate elicited by the seawater, novel predator and herbivore odours (Tukey's HSD: $P < 0.001$ for all three comparisons; Figure 2.2). Similarly, reactions to the herbivore odour did not differ significantly across the three parental treatments, but the mean increase in heart rate of +1.92% differed significantly from those of all the other test cues (Tukey's HSD: $P < 0.005$), except the seawater control. The introduction of a novel predator odour induced similar increases in heart rate (mean = +5.59%; Tukey's HSD: $P = 1.00$), regardless of parental treatment or the predator species from which the novel cue was sourced. Yet,

this reaction to novel predator odours, again, differed significantly from all other test cues (Tukey's HSD: $P < 0.001$; Figure 2.2).

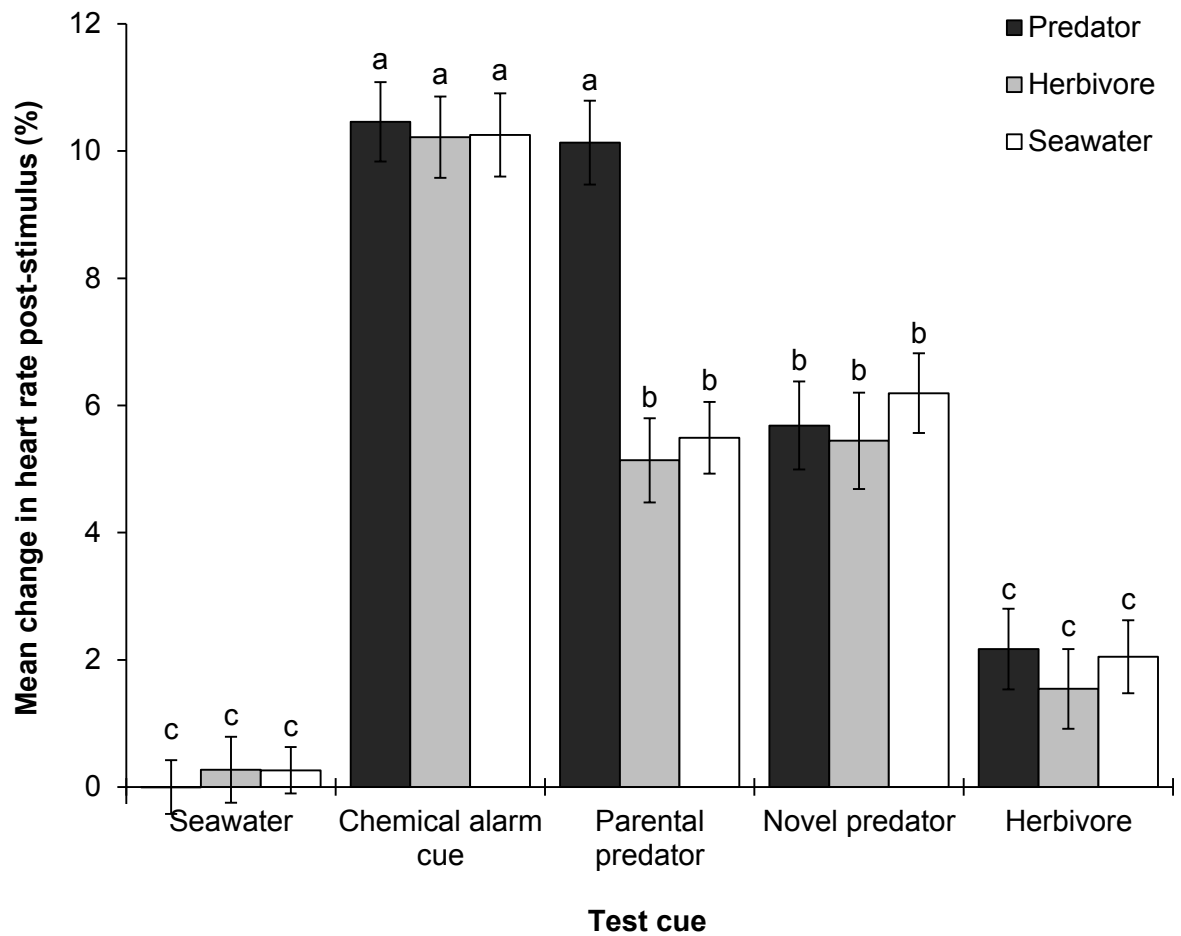


Figure 2.2: Mean change in heart rate (% \pm SE) after introduction of one of the five test cues. Black and grey bars indicate embryos produced by predator-treated and herbivore-treated parents, respectively. White bars indicate embryos produced by seawater treated (control) parents. Groups with the same letter are not significantly different (Tukey's HSD test; $N = 60$ per column).

2.5 Discussion

My findings represent the first example of specific predator information being passed across generations, through non-genetic parental effects. Introduction of a parentally-known predator odour to the vicinity of the eggs induced an almost two-fold increase in heart rate for the offspring from the predator treated parents, compared to the offspring from the herbivore and control parental treatments. As *Acanthochromis polyacanthus*

brood their young for up to three months after hatching (Kavanagh, 2000), the predators experienced by parents are likely to mirror those present in their offspring's environment. The ability of parents to forewarn their offspring of predatory risk has also resulted in a more frequent occurrence of general antipredator behaviours in: three-spined sticklebacks (*Gasterosteus aculeatus*; Giesing *et al.*, 2011), fall field crickets (*Gryllus pennsylvanicus*; Storm and Lima, 2010) and the Tussock skink (*Pseudemoia pagenstecheri*; Shine and Downes, 1999). Additionally, research has demonstrated that parents in high risk environments can increase progeny survival by producing offspring with desirable morphological traits, such as faster growth rates (Besson *et al.*, 2014), and induced defences (Tollrian, 1995). Combined, these phenotypically plastic traits suggest that some parents can gear their offspring to the challenges they are likely to face during early life stages. Still, to my knowledge, this study is the first to demonstrate offspring differentiating between predator cues, showing an increased reaction to a *specific* predator experienced by their parents, and not just a transgenerational response to a risky environment.

However, there is evidence to suggest that parental predator exposure can also have maladaptive consequences for offspring fitness (McGhee *et al.*, 2012). This is likely to be a result of predator presence increasing the concentration of stress hormones, such as cortisol, which can subsequently transfer into the eggs of gravid mothers (reviewed in Green, 2008). Coslovsky and Richner (2012) suggested that if there is a mismatch between the maternal environment and that of the resultant offspring, offspring fitness may suffer as a result of being geared to suit the wrong environment. In the context of the present study, the ability of parents to convey specific predator information to their offspring may provide them with a means for early recognition and escape from predators. Yet, if the conveyed predator information is not pertinent to their offspring's life stage (i.e., due to predator gape limitations [St. John, 1999]), the offspring could incur an energetic cost by reaction to a non-relevant threat (Helfman, 1989). However,

it is possible that any maladaptive effects caused by maternal stress hormones could be overwritten by current environmental conditions (Donelson *et al.*, 2009), or by demonstrating flexibility in growth later in life (Gagliano and McCormick, 2007). For example, Feng *et al.* (2015) demonstrated that by becoming more reliant on social cues, offspring can overcome the reduced learning capabilities caused by maternal stress. This could be particularly pertinent in complex ecosystems, such as coral reefs, where social learning is likely to be very important (Manassa *et al.*, 2013a).

A few mechanisms have been suggested as the means of transgenerational information transfer; namely, hormonal (McCormick, 1998; Groothuis and Schwabl, 2008; Coslovsky *et al.*, 2012) and epigenetic (Youngson and Whitelaw, 2008; Mommer and Bell, 2014), but this is still a relatively new and largely speculative field of research. Due to the specificity of the transgenerational predator recognition observed in this study, we believe the likely mechanism to be the combined effect of hormonal transfer and epigenetic expression. Furthermore, while efforts were made to ensure embryos were not directly exposed to the parental treatments, it is plausible that cues could have been received during gametogenesis (while the eggs were still developing in the mother). These environmental cues could alter the development and behaviour of resultant offspring (Ledón-Rettig *et al.*, 2013), though this has yet to be demonstrated for fishes.

While my results show a clear distinction between reactions to different olfactory cues, the potential adaptive significance of embryos showing tachycardic responses to threat odours is unknown. Research into both aquatic and terrestrial prey species has shown that increases in heart rates often accompany antipredator behaviours and denote predator recognition (Smith and Johnson, 1984; Johnsson *et al.*, 2001). Ydenberg and Dill (1986) also suggested that neurophysiological responses (e.g. changes in heart rate) can provide insight into predator awareness, prior to any observed flight behaviour. Although the existence of embryonic tachycardic responses to threat cues

could imply some form of selective survival benefit (Oulton *et al.*, 2013; Atherton and McCormick, 2015), failing to couple this reaction with a predator avoidance response (e.g., premature hatching, as seen in [Cohen *et al.*, 2016], but not in this study) would still result in increased energy expenditure (Hall and Clark, 2016). Consequently, embryos would be consuming their yolk reserves at a greater rate, incurring a survival cost for larvae upon hatching (Blaxter and Hempel, 1963; McCormick and Nechaev, 2002). In contrast, a study on fishes (Holopainen *et al.*, 1997) found that although exposure to a predatory threat initially induced tachycardia, prolonged exposure resulted in a reduction of overall resting heart rate and activity levels. Steiner and Van Buskirk (2009), found a similar trend in tadpoles, but with oxygen consumption rather than heart rate. In both examples, this long term reduction in metabolic activity in risky environments allowed for more energy to be allocated to growth, which should be beneficial.

There was considerable variability in embryo baseline heart rates and cue induced changes in heart rates, both between and within clutches. However, although the fishes used in this study had been in captivity for a number of years without being exposed to any predator/threat cues, they were originally caught in the wild. Therefore, it is possible that the observed clutch variability could be a result of retention of previously learned predator information. However, although information regarding prior threats can be retained for a period of time without reinforcement, this only tends to last for a period of days/weeks, and only if the predator represented a high threat level (Ferrari *et al.*, 2010c; Ferrari *et al.*, 2010d). Alternatively, the observed disparities could be attributed to an evolutionary phenomenon known as bet hedging, whereby parents produce clutches/offspring which differ markedly phenotypically. In doing so, the parents are producing a range of offspring that are better suited to different environments, increasing the chance of survival of at least part of the cohort, should

the environmental conditions change (Crean and Marshall, 2009; Starrfelt and Kokko, 2012).

My findings also demonstrate that damselfish have innate recognition of predatory threats, indicated by the increase in heart rate induced by a novel predator odour (coral trout and/or dottedyback). In this context, we refer to the 'innate recognition' as a reaction observed in an embryo in response to an olfactory stimulus, which the embryo itself has not previously experienced. While neophobic responses to threat cues are present in some species and situations (Brown *et al.*, 2013; Meuthen *et al.*, 2016), the increases in heart rate seen in this study are unlikely to be a result of neophobia. This is because the embryos showed a significantly greater reaction on introduction of an unknown predator cue compared to the herbivore odour – both of which were 'novel' odours. The seemingly innate ability of prey to recognise a predatory threat by smell could also be a result of the recognition of a common digestive product released by predators after consuming similar prey species (Mirza and Chivers, 2003b; Schoeppner and Relyea, 2005). Innate predator recognition has been identified in other species (Hawkins *et al.*, 2004; Oulton *et al.*, 2013), but this knowledge is often further enhanced through associative learning (Berejikian *et al.*, 2003; Epp and Gabor, 2008; Atherton and McCormick, 2015), or as my findings also suggest, upregulated by parental information and/or experience. Using a combination of mechanisms for recognising predatory threats may be important in life stages and environments with a high risk of predation. As such, when considering the impact predators have on offspring success and population dynamics, a combination of factors, namely parental effects, offspring's own experiences and phenotypes, and genetics, all need to be taken into account (Mommer and Bell, 2013; Stratmann and Taborsky, 2014).

Conclusion

My findings suggest that not only are parents able to convey species-specific predator information to their offspring, but as embryos, offspring also have astute olfactory capabilities with which they can gather further information regarding local threats before hatching. However, further research is required to identify the long term consequences predator induced parental effects have on offspring development, behaviour and survival in later life stages (Chaby, 2016), and determine the mechanism for transfer of predator information in damselfish. Olfactory recognition of predatory threats in embryos could provide a potentially adaptive mechanism for survival, but it seems that post-hatching plasticity may be the key to either overwriting any potential negative consequences of predator induced parental effects or building on any relevant predatory information transferred.

Chapter 3: Active in the sac – damselfish embryos use innate recognition of odours to learn predation risk before hatching

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3.1 Summary

Predation-induced mortality rates of aquatic species are much higher in larvae and juveniles than in adults. Consequently, the ability of an organism to recognise relevant predators as early as possible could increase its chance of survival, especially in areas with high predator diversity. Heart rates of embryonic cinnamon clownfish (*Amphiprion melanopus*) were monitored to assess their reaction to damage-released conspecific alarm cues. These cues were then combined with a predator odour in a conditioning trial to establish if the embryos were capable of learning a predatory threat. Results showed that *A. melanopus* embryos were not only able to detect and react to conspecific chemical alarm cues, but they were also capable of using this information to learn about predation risk before they hatched. This recognition could lead to a number of anti-predation behavioural adaptations, such as modifications of habitat choice at settlement, and affect development and behaviour in post-embryonic individuals, all of which may increase their chance of survival.

3.2 Introduction

Predation is one of the major driving forces in population and community dynamics (Pettorelli *et al.*, 2011). The predators that pose the greatest threat to a prey species can vary greatly with ontogeny and habitat (Wilbur, 1980; Sih *et al.*, 2000; Mitchell and McCormick, 2013). The ability to identify the degree of threat can help individuals to avoid wasting energy on costly anti-predation behaviours; energy which could otherwise be used for other fitness-related activities, such as foraging (Houston *et al.*, 1993; Brown and Smith, 1996). Recognition of predators can be innate (Hawkins *et al.*, 2004), or learned through association using visual and/or chemical cues from predation events (Ferrari *et al.*, 2010a).

Chemical alarm cues (CACs) are odours that are released when the upper epidermis of an animal is damaged. Recognition of such cues is innate and when combined with predator odours, these cues can provide information on relevant threats (Smith, 1992). Research suggests that the epidermal cells containing these alarm cues serve a primarily immune function, and their use as warning signals is a secondary and incidental advantage (Chivers *et al.*, 2007). Nonetheless, both laboratory and field studies have verified the use of chemical alarm cues in learning predation risk in a wide range of aquatic taxa, including amphibians and fishes (Ferrari *et al.*, 2010a; Manassa *et al.*, 2013b). This learning can occur both directly, by witnessing a predation event, or indirectly, through social learning (e.g. observing an individual's anti-predator response to a threat odour (Griffin, 2004). Furthermore, recent research has shown that individuals are able to use alarm cues of conspecifics as well as heterospecifics, and this interspecific learning is also possible in juvenile fishes (Manassa *et al.*, 2013a). After acquiring knowledge about one threat, fishes are able to generalise this information to identify and avoid closely related predators (Ferrari *et al.*, 2007; Mitchell *et al.*, 2013). Most of the research into associative learning of predators using alarm cues has focused on juveniles and adults, especially in fishes, but very little is known

about the sensory and learning capabilities of the early developmental stages, which are most vulnerable to predation.

It is widely accepted in the marine environment that settlement stage fish larvae are naïve to all predators, due to their complex life cycles and pelagic larval stage. Yet, a growing body of research has demonstrated that the predator environment experienced during embryogenesis can induce developmental and behavioural changes, increasing the chance of survival after hatching (Bernard, 2004). For example, common frogs, which experience predators during embryogenesis are shorter with deeper tails, which improves their swimming ability and predator avoidance capabilities as a tadpole (Laurila *et al.*, 2001). Additionally, recognition of predators in early life stages can help individuals differentiate between suitable settlement habitats in coral reef fishes (Vail and McCormick, 2011).

Innate recognition of predators by embryos can also reduce prey mortality through premature hatching. Some frog species have been found to use either vibrational cues (Warkentin, 2005), or chemical cues (Chivers *et al.*, 2001) to hatch early and escape predation from snakes and leeches, respectively. However, although this allows for immediate escape from a predator, there could be subsequent costs, such as an increased chance of mortality in the less developed premature hatchers (Warkentin, 1995; Kusch and Chivers, 2004). Some embryos have also been found to possess the ability to learn predation risk while still in the egg, showing reductions in activity and boldness post-hatching (Mathis *et al.*, 2008; Nelson *et al.*, 2013). There are costs and benefits associated with both innate and learnt recognition of predators, yet a study on the San Marco salamander suggests that some animals could use the combination of the two to exhibit an antipredator response equivalent to the level of threat (Epp and Gabor, 2008).

While the role of CACs in associative learning of predation risk has been widely studied, little is known of its importance to pre-settlement life stages. Tropical reefs are one of the most biodiverse ecosystems in the world, where prey species are often faced with a large variety of predatory threats, from different species with different foraging tactics (Heinlein *et al.*, 2010). Many species lay benthic eggs that are guarded by the parents until hatching, prior to their larval phase. This early association with the parental reef gives them the opportunity to learn about appropriate settlement habitats (Arvedlund *et al.*, 1999; Dixon *et al.*, 2014), but also the potential to learn about predators that may be relevant to the parental habitat. This information is particularly pertinent as recent research has also shown that a large proportion of offspring can return to their natal reef after a one to five week larval phase, and at times settle to sites only metres away from their parents (Berumen *et al.*, 2012). Hence, there may be a selective advantage for fishes to be able to learn about relevant predators as early as possible to avoid being preyed upon. The goal of the present research was to establish the capacity of clownfish embryos to learn a predatory threat. This was achieved by first identifying if the embryos could detect and react to relevant conspecific alarm cues in the latter stages of their embryonic development. Secondly, an associative learning experiment was carried out, whereby embryos were conditioned to recognise a predator odour as a threat, to see if they were able to acquire relevant predator information before they hatch.

3.3 Methods

a) Study species

Amphiprion melanopus is an anemone-associated clownfish species, of the family Pomacentridae, distributed on coral reefs throughout the Western Pacific (Allen, 1991). On average, embryogenesis (egg development) usually lasts for 8 days in this species depending on the temperature, during which time the offspring are well-looked after and nurtured by their parents (Green, 2004). Once they have hatched as transparent

larvae, offspring remain in the pelagic environment for 15 – 21 days before settling on the reef as juveniles (Doherty *et al.*, 1995). *A. melanopus* serves as a good study species for developmental experiments because they successfully reproduce in captivity.

b) Animal housing

All research was carried out, and animals housed, at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University (JCU), Townsville. Five adult breeding pairs of *A. melanopus* were kept outside in 70 L tubs, with half a terracotta pot serving as shelter and a substrate for laying clutches on. These fishes were obtained from an existing broodstock at MARFU, JCU, but were originally wild caught as adults from coral reefs off the coast of Cairns, Queensland, Australia. The tanks were on a constant flow-through system with well-aerated water, which were maintained at 27 °C with a salinity of 35 ppm. Fish were fed pelleted food daily and tanks were then checked for clutches, with the day of spawning being classed as day one post-fertilisation. The eggs were left with their parents until day six post-fertilisation when they were carefully removed from the terracotta pot using a scalpel and collected in a 1 L beaker. The clutch was kept in this beaker overnight, bubbling in very well-aerated water maintained at 27 °C using a flow-through bath.

c) Cue preparation

Chemical alarm cues were made by crushing ten *A. melanopus* embryos, from the same clutch as the test embryos, in a petri-dish, and rinsing them with 5 ml of seawater. This was then filtered through filter paper to remove any particulate matter, leaving only seawater infused with alarm cue. One ml was used per trial, and fresh embryo alarm cues were made as required throughout the experiments, to prevent degradation of cues.

Predator odours were prepared using the dottyback, *Pseudochromis fuscus*, a well-known opportunistic predator, which preys on both eggs and juvenile damselfish (Emslie and Jones, 2001; Feeney *et al.*, 2012). A 10 cm (total length), *P. fuscus* individual was starved for 24 h prior to the experiment to limit the amount of dietary cues used in trials. They were then placed in 10 L of well-aerated seawater for twelve hours before any cues were collected. Water was removed for each set of five trials, to reduce the chance of degradation of cues. Once trials had been run for the day, a complete water change was undertaken to reset the concentration of the odour used in the trials carried out the next day (day eight post-fertilisation).

d) Embryonic detection of chemical alarm cues

A time series trial was carried out to examine whether or not *A. melanopus* embryos were able to detect and respond to alarm cues. On day five post-fertilisation, embryos were carefully removed from the clutch and kept in 1 L, well-aerated beakers overnight at the same temperature as the parental tanks. On day six, embryos were tested with either seawater (SW) or an embryo chemical alarm cue stimulus (CAC; $N = 15$ embryos per cue). This was carried out by placing an embryo in a small container of 10 ml of seawater, under a dissecting microscope and allowing it to acclimatise for 2 min. The baseline heart rate (beats per 30 s), was then measured using a stopwatch and a tally counter. The cue (1 ml) was then added to the seawater and the heart rate was re-measured, to allow the calculation of any stimulus-induced changes in heart rate. On day seven, different embryos from the same clutch were tested with the same, freshly prepared chemical alarm cue stimulus to assess if there was a difference in reaction to the odour with a further 24 h development. This experiment was repeated using four different clutches produced by four different breeding pairs.

e) Associative learning in embryos

(i) Day 7 treatment trials

Previously untested embryos from each clutch were split across one of three treatments on day seven post-fertilisation (Table 3.1): (i) seawater control (SW), (ii) predator odour (PO), or (iii) predator odour combined with a chemical alarm cue (PO+CAC). A subsample of fifteen embryos was taken to assess the change in heart rate induced by each of the three test cues. Heart rate (per 30 s) was recorded before and after stimulus introduction, and the percentage difference was calculated for each embryo, using the same methods outlined in the previous experiment. The proportions of the three cues were as follows: (i) 2 ml of seawater, as a control; (ii) 2 ml of predator odour; (iii) 1 ml of predator odour and 1 ml of an embryo chemical alarm cue.

A further 30 embryos from each clutch were treated (but not tested) per cue to account for any potential overnight mortality. Thus, there were a total of 45 embryos, for each treatment, kept in well-aerated beakers in water baths overnight, to be re-tested on day eight post-fertilisation. This experiment was repeated for four separate clutches, produced by four different breeding pairs of *A. melanopus*.

(ii) Day 8 test trials

On the following day (day eight post-fertilisation), fifteen embryos from each of the three day seven treatments were tested with 1 ml of predator odour (Table 3.1). The same procedure was carried out on day seven was followed to establish each embryo's reaction, in terms of their change in heart rate induced by the cue. Therefore, the three sets of treatments undergone by the subsamples of embryos were: (i) seawater control, then predator odour; (ii) predator odour, then predator odour again; (iii) predator odour with chemical alarm cue, followed by predator odour alone. This allowed us to assess if *A. melanopus* embryos are able to use chemical alarm cue to learn, through conditioning, to recognise predation threat.

Table 3.1: Experimental design for the embryo treatments and trials examining associative learning in embryos.

	Cues introduced		
	Treatment 1	Treatment 2	Treatment 3
Day 7 – treatment trials	Seawater	Predator odour	Predator odour + Embryo alarm cue
Day 8 – test trials	←———— Predator odour —————→		
Behavioural phenomena being tested	Experimental control	Neophobic response	Associative learning

f) Statistical analyses

Residual analysis found that data met the assumptions of ANOVA. The model used for the pilot trial tested cue and clutch, and the interaction between them as fixed factors. Similarly, the model for the learning experiment analysed looked at the effects of fixed parameters clutch, day and treatment, and the interactions among them. Tukey's HSD post-hoc tests were used to see where the significant differences lay in both datasets.

g) Ethical note

All research was approved by the James Cook University Animal Ethics Committee under the permit A1871.

3.4 Results

a) Embryonic detection of chemical alarm cues

The ANOVA showed a significant difference between the increases in heart rate induced by SW and CACs on day 6 and 7 post-fertilisation ($F_{2, 329} = 16.659$; $P < 0.0001$; Table 3.2). Furthermore, clutch did not have a significant impact on mean heart rate increase ($F_{3, 168} = 0.587$; $P = 0.624$). Introduction of a SW control on day 6 induced a

negligible change in heart rate, which was significantly different from those induced by both CAC treatments (SW day 6 = -0.07%; Tukey's HSD: $P < 0.0001$; Figure 3.1). Embryos exhibited a greater reaction to a conspecific CAC (in terms of an increase in heart rate) on day 7 of development, compared to those on day 6 (day 6 = +6.60%, day 7 = +12.22%; Tukey's HSD: $P < 0.0001$).

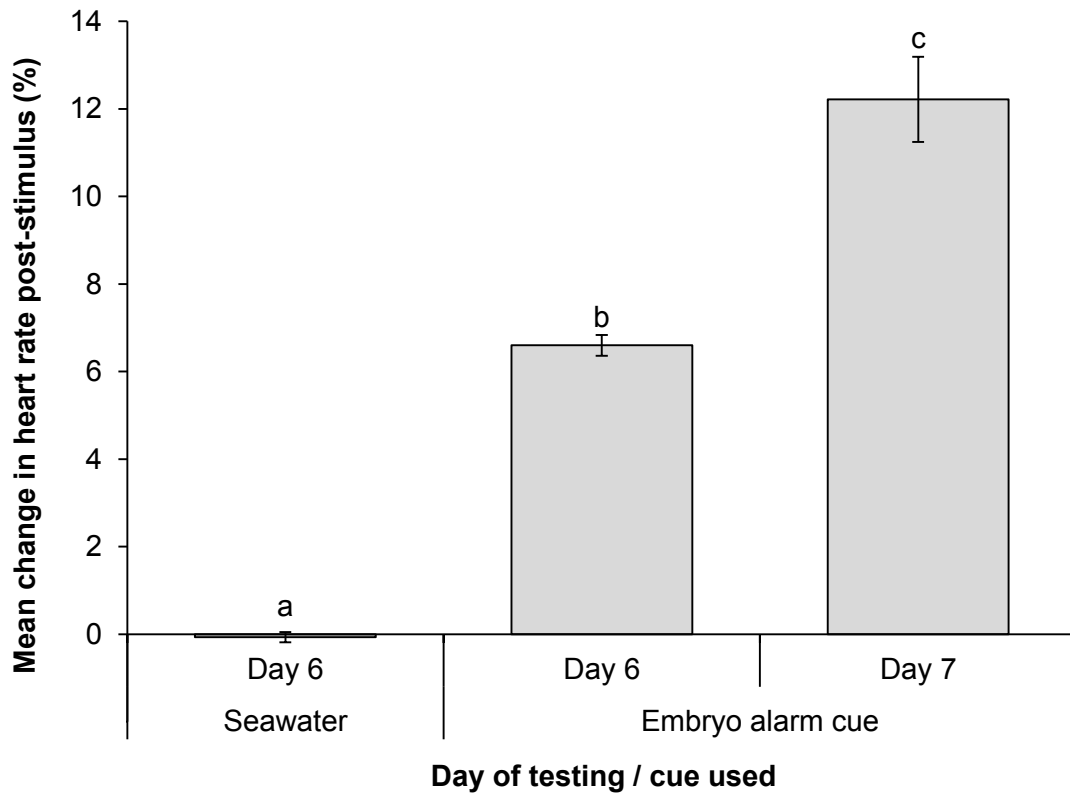


Figure 3.1: Comparison of the mean percentage change in heart rate (\pm SE) induced by a within-clutch embryo alarm cue on day six and day seven of post-fertilisation development in *Amphiprion melanopus* embryos, compared to a seawater control ($N = 60$ per treatment). The letters above the bars represent Tukey's HSD groupings of means.

In order to show that it was not the introduction of a cue on consecutive days that induced a stress response (increase in heart rate), a pilot study testing embryos with SW cues on both day 7 and day 8 was conducted. SW cue caused a 0.18% increase in heart rate on day 7, followed by a 0.10% increase on day 8. An ANOVA showed this to be a non-significant difference ($F_{2, 54} = 0.190$; $P = 0.828$; Table 3.3).

Table 3.2: A two-factor ANOVA comparing the change in heart rate of embryonic *Amphiprion melanopus*, induced by the type of cue used (seawater or conspecific alarm cue) and the day of testing (day 6 or 7 of development), and the clutch from which the embryos were sourced.

Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Clutch	3	9.781	0.587	0.6241
Cue and Day	2	2270.034	136.323	<0.0001
Clutch * Cue and Day	6	25.734	1.545	0.1663
Error	168	16.652		

Table 3.3: A two-factor fixed ANOVA comparing the change in heart rate induced by a seawater cue on day 7, followed by a repeated seawater cue on day 8, and the clutch from which the embryos were sourced.

Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Clutch	2	0.012	0.001	0.9992
Day	1	0.084	0.006	0.9394
Clutch * Day	2	2.748	0.190	0.8276
Error	54	14.468		

b) Associative learning in embryos

An ANOVA showed the interaction between day and treatment cue was significant ($F_{2,329} = 16.659$; $P < 0.0001$). All other factors, and interactions between effects, of clutch, day and treatment cue, were not significant (Table 3.4). When treated with a PO combined with a CAC during the initial conditioning, embryos showed an increase in heart rate of +7.91%. This increase was significantly higher than that produced by PO (Tukey's HSD: $P < 0.0001$), or SW conditioning ($P < 0.0001$). When the PO+CAC conditioned fish were re-tested with just a PO on day eight, the conditioned embryos

showed a very similar increase in heart rate as they did on day seven (+8.07%; Tukey's HSD: $P = 0.999$; Figure 3.2), suggesting they had learnt that PO represented a threat.

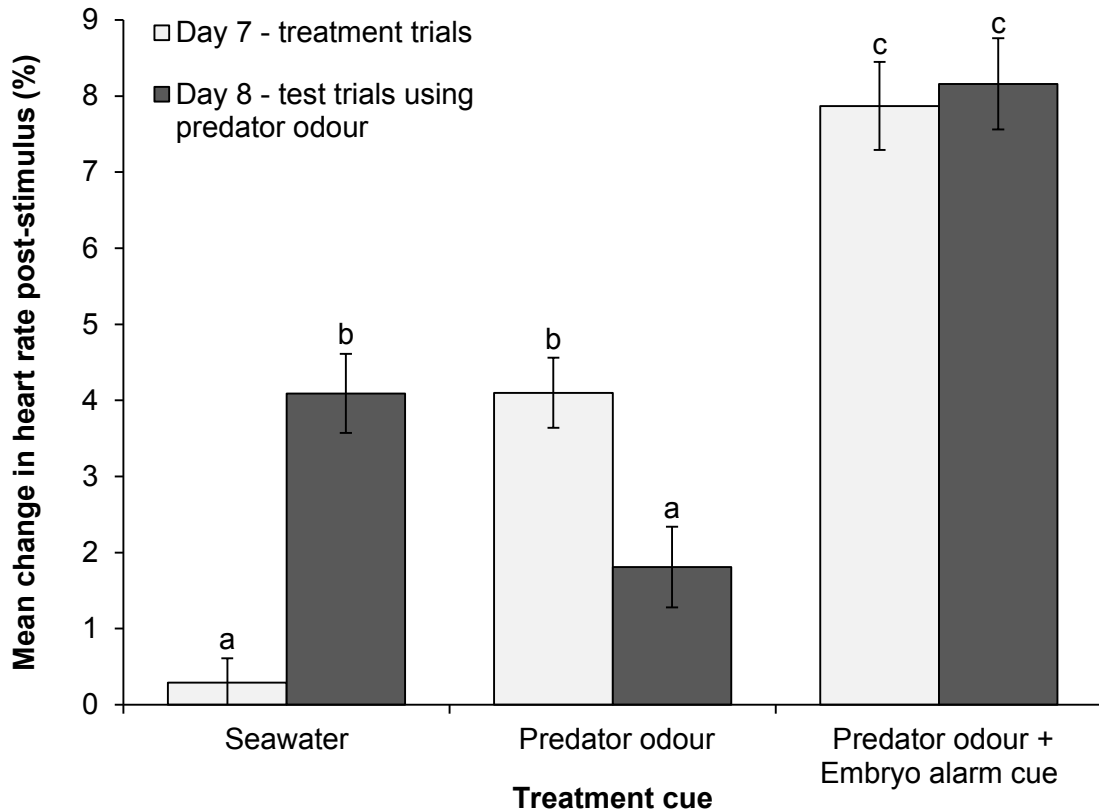


Figure 3.2: Mean percentage change in heart rate (\pm SE) after cue introduction. The white bars indicate the reaction to one of three treatment cues on day seven of development: seawater, predator odour, or predator odour combined with a within clutch embryo chemical alarm cue. The dark grey bars indicate the increase in heart rate induced by the introduction of the predator odour to embryos from each of the three conditioning treatments, on day eight of development. The letters above the bars represent Tukey's HSD groupings of means ($N = 60$ per column, except the dark grey bar in predator odour treatment, where $N = 53$).

Embryos tested with PO, both initially as a treatment on day seven and on day eight (after SW conditioning on day seven), showed very similar increases in heart rates post-cue introduction (PO day 7 = +4.10%, SW day 7-PO day 8 = +4.09%; Tukey's HSD: $P = 1.000$). The increase in heart rate induced by PO on day seven was significantly larger than that of the SW control, which elicited very little response (SW day 7 = +0.29%; Tukey's HSD: $P < 0.0001$).

Table 3.4: A three-factor ANOVA investigating the changes in *A. melanopus* embryo heart rates in response to the treatment cue used (seawater, predator odour or predator odour + conspecific alarm cue) during and after a conditioning event, while taking into account the source of the clutch.

Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Clutch	3	6.620	0.419	0.7394
Day	1	30.762	1.948	0.1638
Treatment cue	2	1172.841	74.254	<0.0001
Clutch * Day	3	3.131	0.198	0.8956
Clutch * Treatment cue	6	3.686	0.233	0.9655
Day * Treatment cue	2	263.134	16.659	<0.0001
Clutch * Day * Treatment cue	6	3.299	0.209	0.9739
Error	329	15.795		

Embryos that were treated with a PO on day seven, and then re-tested with PO on day eight, showed a significantly reduced increase in heart rate with cue introduction (PO day 7 = +4.10%; PO day 7-PO day 8 = +1.81%; $P = 0.020$). However, although a slight increase in mean heart rate was still induced by the day eight PO test cue, the change in heart rate was not statistically different from that of the SW control (Tukey's HSD: $P = 0.291$).

3.5 Discussion

Embryos of clownfish are able to detect and react to chemical alarm cues of conspecifics. The increase in heart rate induced by the presence of a chemical alarm cue suggests recognition is innate and can occur immediately after olfactory development. Furthermore, the two-fold increase in reaction from day 6 to day 7 suggests that olfactory senses develop rapidly in the latter stages of embryogenesis. This is supported by research carried out by Arvedlund *et al.* (2000) who found that

olfactory development starts on day 6 post-fertilisation, but embryonic olfaction is unlikely to be fully functional until day 7. Kavanagh and Alford (2003) also found that olfactory development occurred more quickly in an anemonefish than in other pomacentrids (black axil chromis, ambon damsel and spiny chromis). It has been suggested that this rapid olfactory development may allow clownfish to imprint on their host anemone (Arvedlund and Nielsen, 1996).

The clownfish embryos were able to use their innate recognition of CACs to learn to recognise a correlate of predation risk. A positive correlation between increases in heart rate and antipredator behaviours has been reported in a number of species of fishes (Metcalf *et al.*, 1987; Höjesjö *et al.*, 1999; Johnsson *et al.*, 2001). With this in mind, it is likely that the increase in heart rate exhibited by the conditioned *A. melanopus* embryos could later translate to increased predator avoidance in settlement stage juveniles, through enhanced recognition of risk. However, an embryonic tachycardic response would also increase energy expenditure (Hall and Clark, 2016), and thus, utilise more of the allotted yolk reserve, resulting in a possible survival disadvantage upon hatching (Blaxter and Hempel, 1963; McCormick and Nechaev, 2002). The learning capability of embryos has only been identified in a couple of other aquatic species: wood frogs and ringed salamanders (Mathis *et al.*, 2008), convict cichlids (Nelson *et al.*, 2013) and rainbowfish (Oulton *et al.*, 2013). Individuals with knowledge of relevant predatory threats experience higher survival rates (Mirza and Chivers 2001a; Lönnstedt *et al.*, 2012a; Polo-Cavia and Gomez-Mestre, 2014). In spite of this, it is widely thought that new coral reef fish recruits are naïve to predators at settlement stage. As coral reefs have high biodiversity, juveniles are subject to intense predation pressure from a suite of predators once they have hatched (Almany and Webster, 2006). Mitchell *et al.* (2011a) showed settlement-stage juvenile lemon damselfishes (*Pomacentrus moluccensis*) were able to quickly learn a number of novel predators using chemical stimuli, and exhibit anti-predator responses to odours after a

single conditioning event. My study suggests that some species of damselfish are capable of doing this at the embryonic stage and may in fact already have some knowledge of relevant predatory threats when they hatch.

Research suggests that some species of fish may have innate predator recognition (Dixon *et al.*, 2010; Kempster *et al.*, 2013; Oulton *et al.*, 2013). The increase in embryo heart rate induced by the predator cue in my experiment could be interpreted as such (Oulton *et al.*, 2013). However, there is also the possibility that this reaction could be neophobic; i.e., a 'fear' response induced by an unfamiliar cue (Brown *et al.*, 2014). Embryos that were treated with a predator odour twice demonstrated a reduced reaction to the cue on the second exposure. This suggests their first reaction could be a neophobic response, as without the reinforcement of a predation event/alarm cue, the perceived threat level of the predator odour decreased. Berejikian *et al.* (2003) studied naïve, hatchery-reared chinook salmon and found an innate anti-predator response, which was significantly amplified by a conditioning event with an alarm cue. Thus, while it may be possible that some fishes possess innate predator recognition, it is likely that the continuation of anti-predator behaviours to such cues would cease if not associated with an alarm cue (Mitchell *et al.*, 2011b).

Once an individual has learnt to recognise a threat, this information could be used to avoid predation during subsequent ontogenetic stages. Hepper and Waldman (1992) showed that a preference for a chemical stimulus experienced as an embryo lasted through the larval stage, metamorphosis and into the juvenile stage in two frog species. This finding was supported by more recent studies, in which embryonic conditioning with alarm cues prompted increased anti-predator behaviours post-hatching in wood frogs, ringed salamanders (Mathis *et al.*, 2008) and convict cichlids (Nelson *et al.*, 2013). Additionally, learned predator information can be retained by prey species, even without continual reinforcement, depending on the level of risk that predator represents (Ferrari *et al.*, 2010c; Ferrari *et al.*, 2010d). Research has shown that levels of

dispersal in anemonefish are a lot lower than was first thought, with high levels of self-recruitment to parental reefs (Jones *et al.*, 2005). Therefore, predators experienced by individuals in the embryonic stage are likely to be similar and relevant to those experienced by many settlement stage larvae and juveniles. Indeed, many of the key predators on newly settled juvenile fishes also eat embryos from benthic egg clutches, such as the moonwrasse (*Thalassoma lunare*) and dottyback (*Pseudochromis fuscus*) (McCormick and Meekan, 2007). Hence, the chance of mortality through predation is likely to be decreased in settlement stage fishes that have prior knowledge of the predators in their microhabitat.

My findings indicate that embryonic anemonefish have functional olfactory receptors capable of detecting chemical stimuli important in early life stages. Numerous studies have shown clownfish innately imprint on certain host species of anemones, which strongly influences their selection of settlement habitats (Arvedlund and Nielsen, 1996; Arvedlund *et al.*, 1999 and 2000; Dixon *et al.*, 2008). It has already been shown in a number of taxa, that juveniles use chemical recognition to avoid settling in habitats where predators are present; i.e., barnacles (Johnson and Stratmann, 1989); plaice (Wennhage and Gibson, 1998); salamanders (Mathis *et al.*, 2008), and fishes (Vail and McCormick, 2011). Therefore, it is possible that the rapid development of olfaction and early learning capabilities of clownfish could increase survival through informed habitat choice and avoidance of predators. Dixon (2012) recently examined the combined effect of predator and host/non-host anemone (species specific) odours on habitat selection in three species of clownfish, and showed that larvae strongly prefer host anemones, especially in the absence of predators; demonstrating the importance of odour identification and categorisation.

Conclusion

My findings suggest that anemonefish embryos have refined olfactory capabilities before they hatch and can use chemical stimuli to learn predatory threats during embryogenesis. This could aid survival through predator avoidance by selecting safer habitats at settlement and refining predator recognition to prevent wasting energy on anti-predator behaviours. Hence, it would be important to determine whether predator information learned as an embryo carries through the pelagic larval stage in fishes with a bipartite life cycle. Additionally, further research is needed to fully assess the extent of the embryonic sensory and learning capabilities of fishes and the impact they have on subsequent life stages.

Chapter 4: Save your fin, listen to your kin – promoting survival through kin recognition

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4.1 Summary

Early recognition of relevant threats can promote survival by allowing individuals to only carry out antipredator behaviours when cues signifying a true threat are released, and responding to odours in a threat sensitive manner can conserve energy. Recognition of, and cooperation with, kin can improve foraging and promote more efficient antipredator responses, protecting the gene pool and increasing individuals' inclusive fitness. The seemingly altruistic act of emitting damage-released alarm cues during predatory attacks is thought to be a potential driver of kin recognition. By observing changes in heart rates, I demonstrated that embryos of two damselfish species (*Acanthochromis polyacanthus* and *Amphiprion melanopus*) not only possess innate recognition of kin and damage-released alarm cues, but also react to them in a graded manner. Such refined olfactory capabilities in embryonic stage organisms suggest identification of threats may provide survival advantages post-hatching, such as the informed choice of low risk habitats at settlement.

4.2 Introduction

Kin recognition is the ability of organisms to distinguish their relatives from other conspecifics and has been identified in a wide range of taxa. Examples include: amphibians (Blaustein and Waldman, 1992), birds (Krause *et al.*, 2012), fish (Frommen *et al.*, 2013), insects (Whitehorn *et al.*, 2009), mammals (Mateo, 2003), and reptiles (Léna and Fraipont, 1998). Kin identification reduces problems associated with inbreeding and enables differential behavioural interactions that promote fitness of kin, thereby increasing inclusive fitness (Hamilton, 1964; Frommen *et al.*, 2007). Many factors affect the presence and extent of kin recognition in a species, as well as the mechanisms through which it is achieved (Waldman, 1988). For example, schooling species show a greater propensity for kin recognition, as they are more likely to encounter relatives throughout their lives (Carreno *et al.*, 1996; Arnold, 2000). Kin recognition may also assist individuals in avoiding predation (Keogh, 1984; FitzGerald and Morrisette, 1992; Brown, 2002; Griffiths *et al.*, 2004) through the production of warning signals. When living in close proximity to kin, the seemingly altruistic act of releasing alarm or disturbance cues to alert family members of local predatory threats can improve their chance of evading capture, thereby increasing their inclusive fitness (Smith, 1986; Blaustein, 1988; Wisenden and Chivers, 2006).

Chemical alarm cues (CAC) are only released when the upper epidermis of an aquatic organism is damaged (Chivers and Smith, 1998), meaning they are an honest and reliable indicator of a threat. As the predators that pose a threat to prey species can vary greatly with both life history and habitat (Wilbur, 1980), it can be imperative to survival to be able to not only recognise CAC, but also know which are relevant (Hill and Weissberg, 2014). Recognition of both conspecific and heterospecific cues can be innate, implying that it confers a survival advantage in early life (Brown *et al.*, 2011a). Mitchell *et al.* (2012) found the magnitude of the antipredator behaviour of a damselfish species elicited by CAC directly related to the phylogenetic proximity of the donor.

Research suggests that individuals often react in a graded manner to chemical alarm cues, depending on the level of relevant risk they represent (i.e. higher concentration of the cue, and cues from the same ontogenetic stage represent greater threat; Vavrek and Brown, 2009; Ferrari and Chivers, 2010; Mitchell and McCormick, 2013).

Embryos have been shown to possess advanced olfactory capabilities. Not only can they detect and react to risk odours, but some can associatively learn risk from odours and respond to these in a threat sensitive manner (Mathis *et al.*, 2008; Ferrari and Chivers, 2010; Ferrari *et al.*, 2010b; Oulton *et al.*, 2013; Atherton and McCormick, 2015; **Chapter 5**). It is currently unknown whether embryos are capable of differentially prioritising information from kin with respect to threat relevance. Such information should be advantageous as it provides the most relevant information on threats in the immediate vicinity of the individual.

The present research sought to determine if embryonic fishes had innate recognition of kin and damage-released cues, and whether they responded differentially to cues from donors of varying levels of relatedness and phylogenetic proximity. Changes in the heart rate of two species of coral reef damselfish (*Amphiprion melanopus* and *Acanthochromis polyacanthus*) were assessed in response to conspecific cues originating from three sources: (i) kin – siblings from the same clutch; (ii) kin previous – offspring from the same parents, but from a different clutch; and (iii) non-kin – conspecifics from a different breeding pair. Additionally, I tested the reaction of embryos from both species to two different heterospecific cues. Both species were tested against cues from a phylogenetically different damselfish (*Chrysiptera cyanea*), and *A. melanopus* and *A. polyacanthus* were tested against cues from embryos of the other species, representing a phylogenetically closer heterospecific (Cooper *et al.*, 2009). My findings demonstrate sophisticated olfactory capabilities in embryos of both species of damselfish, whereby they innately recognised and differentiated between kin and non-kin conspecifics, and heterospecifics, and responded in a graded manner.

Additionally, although it is often considered to be an innate mechanism, this is the first time that kin recognition has been identified in embryos of any species.

4.3 Methods

a) Study species

The present study compares the embryonic recognition of cues in two species, one of which has a conventional dispersive larval phase (*Amphiprion melanopus*), while the other species is the only damselfish to brood its young (*Acanthochromis polyacanthus*). Kin selection is potentially relevant to both species, as for those species with a pelagic larval phase, studies have found a high proportion of larvae returning (~ 50%) not only to their natal reef, but settling within metres of their parents (Jones *et al.*, 1999; Jones *et al.*, 2005; Berumen *et al.*, 2012). For *Acanthochromis polyacanthus*, juveniles stay with the parents for around three months after hatching (Thresher, 1985; Kavanagh, 2000) and then disperse locally to varying extents (Doherty *et al.*, 1994; Miller-Sims *et al.*, 2008). This means interactions amongst kin may be important in affecting early survival when they are most vulnerable to predators (Almany and Webster, 2006).

Amphiprion melanopus is a coral reef associated damselfish, and a member of the family Pomacentridae (Subfamily: Amphiprioninae; more commonly known as clownfishes). *A. melanopus* are an anemone-associated species and have a relatively short pelagic larval stage for a damselfish, at just 15 – 22 days (Doherty *et al.*, 1995). The eggs are usually around 2.3 mm in length and they develop for 7.5 days before hatching as transparent larvae, in the evening, and disperse into the pelagic environment (Green, 2004).

In contrast, *Acanthochromis polyacanthus* (Pomacentridae) is the only reef associated damselfish species to lack pelagic larval stage. The parents brood their offspring for up to three months after hatching, before they disperse elsewhere (Kavanagh, 2000). Embryogenesis usually lasts 9 – 11 days (Donelson *et al.*, 2008), and *A. polyacanthus*

eggs are very large compared to those of other damselfish (around 4 mm in length). They hatch during the day, and are considered to be settled at hatching (Kavanagh and Alford, 2003).

Many coral reef fishes have very small home ranges and the identity of predators can be highly variable on a small spatial scale, so survival of juveniles is often dependent on their knowledge of local threats. Studies that have manipulated the provision of information concerning the risk from specific species of predators using CAC mediated associative learning have highlighted the marked survival benefits to the early provision of risk information (Lönnerstedt *et al.*, 2012a; Manassa and McCormick, 2012).

b) Animal housing

All animals used were housed and experiments were carried out at the Marine and Aquaculture Research Facilities Unit, James Cook University. Adult breeding pairs of both *A. polyacanthus* (7 pairs) and *A. melanopus* (3 pairs) were kept in well-aerated 70 L tubs of seawater, on a constant flow-through system. The temperature was maintained at 28 °C throughout the breeding season, with a salinity of 35 ppm. The holding tanks were situated outside, so the fish were kept under a normal summer diurnal light cycle (light:dark, 12.5:11.5 h). Half of a terracotta pot was placed in each tank for the breeding pairs to use as shelter and to provide a surface for the females to lay their eggs on. Adults were fed pelleted food twice per day, at which point all tanks were also checked for egg clutches.

When a clutch of eggs was produced, it was left with its parents until two days prior to hatching; this occurred at day six after fertilisation for *A. melanopus* and ten days post-fertilisation for *A. polyacanthus*. Embryos were collected from their parental tanks by gently cutting the tissue adhering the clutch to the terracotta pot with a scalpel. Each clutch of embryos was kept in a separate well-aerated 1 L tub, in a shared water bath with flow through water, to maintain the temperature at that of the parental tanks. Water

changes (25%) were carried out twice daily, using water from the parental tanks and the light cycle in the laboratory was set to mirror that of the outside parental tanks.

c) Stimulus preparation

The response of embryos was recorded to a seawater control, or one of five damage-released cues from eggs that included: (i) embryos of kin, (ii) kin from a previous clutch, (iii) non-kin conspecifics, (iv) *A. melanopus* or *A. polyacanthus* (the opposite species to the recipient, (v) *Chrysiptera cyanea*. The seawater for the control cue trials was sourced from the respective parental tanks. All cues derived from *A. polyacanthus* embryos were created by crushing five embryos in a petri dish, and then adding 5 ml of seawater. This solution was then passed through filter paper, removing any particulate matter to leave the cue-infused seawater, which provided enough for five replicate trials. The same method for cue production was used for all *A. melanopus* cues, the only difference being that ten embryos were used instead of five to produce 5 ml of cue. This is because *A. polyacanthus* embryos are almost twice the size of those of *A. melanopus*.

C. cyanea eggs are very small in comparison to the other two damselfish species (around 1.3 mm; Gopakumar *et al.*, 2009). Thus, the quantity of eggs used to make each set of five *C. cyanea* CAC was determined by weighing five *A. polyacanthus* embryos and ten *A. melanopus* embryos and using the mean as a guide for portioning off a standardised weight range for the *C. cyanea* eggs (Table 4.1). Once weighed, the *C. cyanea* embryos were promptly placed into vials and frozen in liquid nitrogen. A pilot trial was carried out which showed that a very similar magnitude of heart rate increase was induced by damage-released cues produced by both fresh and frozen (and defrosted) embryos (see Appendix 2: Chapter 4 pilot trial). This facilitated the measurement of the response of embryos to CAC produced from embryos from the same parents, but from a previous clutch (kin previous).

Table 4.1: Mean mass (mg) of eggs from three species of damselfish, calculated from five replicated measurements of dry weight.

Species	Number of eggs	Mean mass (mg)
<i>Acanthochromis polyacanthus</i>	5	40
<i>Amphiprion melanopus</i>	10	36
<i>Chrysiptera cyanea</i>	63 (mean)	38

d) Experimental procedure

Trials were carried out on the day of hatching for *A. melanopus* and the day before hatching for *A. polyacanthus*. Change in heart rate was determined in reaction to one of the six olfactory cues previously outlined for both species. For each trial, one embryo was carefully placed in a small container of 10 ml of seawater, which was then positioned under a dissecting microscope illuminated with a cold light. The embryo was allowed to acclimatise for 2 min, after which the heart rate was recorded for 30 s. One ml of one of the six test cues was slowly injected into the container, and the heart rate was recorded for another 30 s.

Changes in heart rate have been identified as a viable behavioural proxy for quantifying reactions to predatory threats, as increases or decreases in heart rate often simultaneously accompany, or precede, antipredator behaviours (Ydenberg and Dill, 1986; Höjesjö *et al.*, 1999). With fluctuations in basal heart rates and tachycardic responses to threats being shown to induce phenotypical plasticity traits that infer a survival advantage (Holopainen *et al.*, 1996), changes in heart rate are thought to be a valid means to assess threat recognition in embryos which are unable to respond with any other observable behaviour (Oulton *et al.*, 2013; Atherton and McCormick, 2015).

e) Statistical analyses

Two linear mixed-effects ANOVA models tested cue type as a fixed factor, but also included clutch as a random factor, to determine if the changes in embryo heart rates differed with the type of cue introduced; one for *A. melanopus* and one for *A. polyacanthus*. Tukey's HSD post-hoc tests were used to determine where the significant differences lay in the data for each species.

f) Ethical note

All experiments were approved by the James Cook University Animal Ethics Committee under the permit A1871.

4.4 Results

There were significant differences between the changes in heart rates induced by the six test cues for both species (*Amphiprion melanopus* and *Acanthochromis polyacanthus*; Table 4.2). In *A. melanopus* the chemical alarm cues (CAC) derived from both direct kin (+10.50%) and kin from a previous clutch (+9.13%) caused an increase in heart rate that was significantly larger than all other CAC (Tukey's HSD: $P < 0.05$ for all interactions; Figure 4.1). The same significantly larger reaction to both types of kin CAC (kin = +11.32%; kin previous = +10.02%) occurred in *A. polyacanthus* (Tukey's HSD: $P < 0.01$ for all pairwise comparisons; Figure 4.2). For both species, the introduction of a seawater control cue induced negligible increases in heart rate (*A. melanopus* = +0.06%; *A. polyacanthus* = +0.08%), which contrasted markedly from CAC derived from conspecifics (Tukey's HSD: <0.001 for all; Figures 4.1 and 4.2).

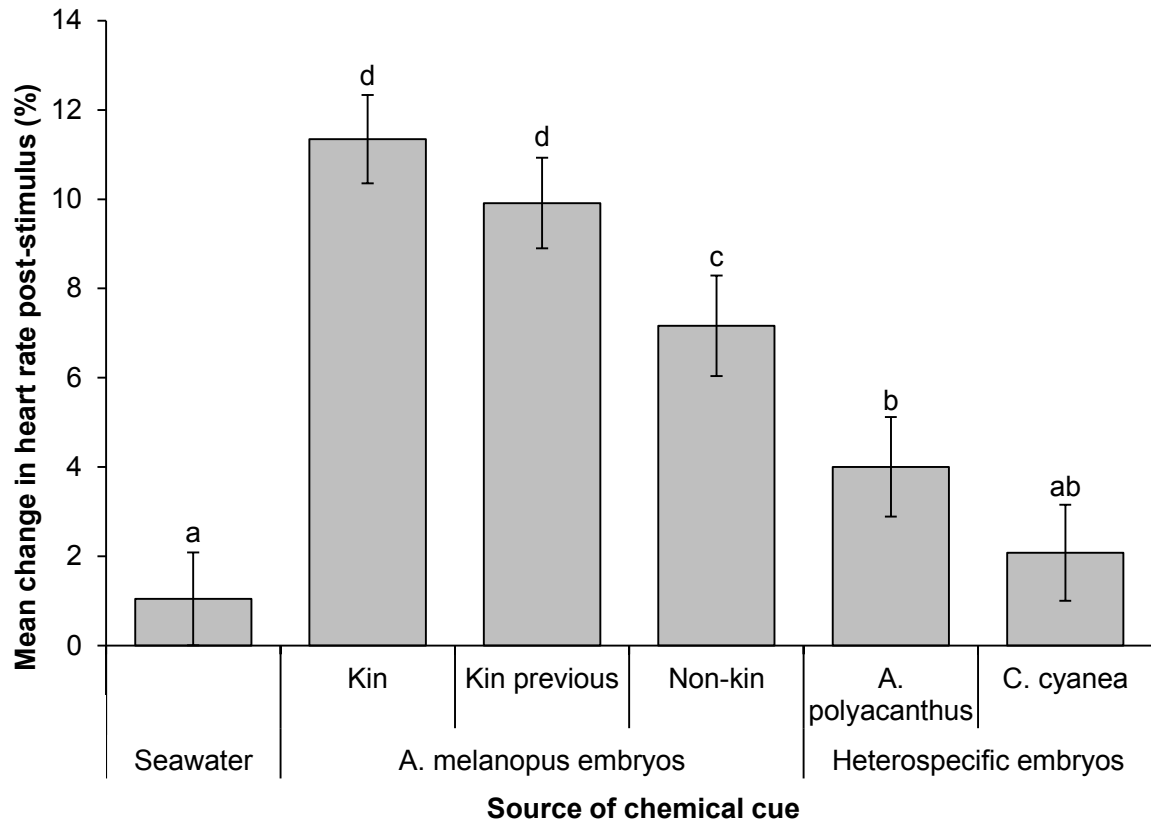


Figure 4.1: Mean percentage change in heart rate (\pm SE) of *Amphiprion melanopus* embryos after introduction of one of six chemical cues (saltwater, or conspecific alarm cues from: direct kin, kin from a previous clutch, or non-kin; or heterospecific alarm cues from: *Acanthochromis polyacanthus*, or *Chrysiptera cyanea*). Letters indicate Tukey's groupings of means ($N = 75$ for seawater and kin cues, and $N = 45$ for the remaining four cues).

The introduction of a conspecific, non-kin cue provoked a significantly lower increase in heart rate compared to conspecific kin CAC in both *A. melanopus* (+6.33%; Tukey's HSD: $P < 0.05$) and *A. polyacanthus* (+5.85%; Tukey's HSD: $P < 0.01$). Heterospecific cues (either *A. melanopus* / *A. polyacanthus*, or *C. cyanea*) induced increases in heart rates for both species; however, these increases were significantly lower than the reactions elicited by any of the three conspecific cues (Tukey's HSD groupings on Figures 4.1 and 4.2).

Table 4.2: Results of two, linear mixed effects ANOVAs (with clutch included as a random factor) investigating the comparison of the mean changes in heart rates induced by one of six chemical alarm cues, in two damselfish species: *Amphiprion melanopus* and *Acanthochromis polyacanthus*.

Species	Effect	df	MS	F	P
<i>Amphiprion</i>	Cue	5	1155.967	68.535	< 0.0001
<i>melanopus</i>	Residual	324	16.867		
<i>Acanthochromis</i>	Cue	5	1843.822	72.670	< 0.0001
<i>polyacanthus</i>	Residual	424	25.373		

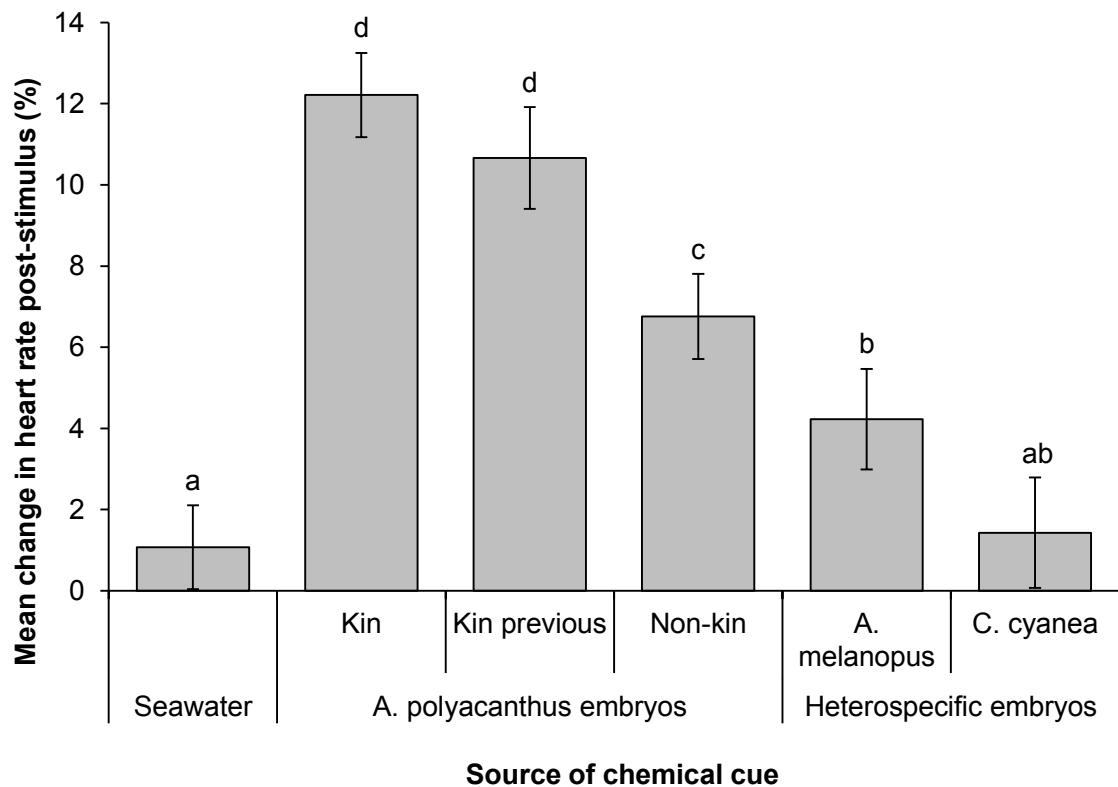


Figure 4.2: Mean percentage change in heart rate (\pm SE) of *Acanthochromis polyacanthus* embryos after introduction of one of six chemical cues (saltwater, conspecific alarm cues from: direct kin, kin from a previous clutch, or non-kin; or heterospecific alarm cues from: *Amphiprion melanopus*, or *Chrysiptera cyanea*). Letters denote Tukey's groupings of means (left to right, N = 120, 120, 30, 75, 55 and 30, respectively).

4.5 Discussion

Embryos of both species of damselfish (*Amphiprion melanopus* and *Acanthochromis polyacanthus*) were able to differentiate between kin and other non-related conspecifics, and heterospecifics, using olfactory cues. The ability of juveniles and adults to recognise their kin using olfactory cues alone has been identified in a limited number of taxa, including freshwater fishes (three-spined sticklebacks, *Gasterosteus aculeatus*; Mehlis *et al.*, 2008; and zebrafish, *Danio rerio*; Mann *et al.*, 2003), and birds (zebra finches, *Taeniopygia guttata*; Krause *et al.*, 2012), but never at the embryonic stage. Blaustein *et al.* (1984) demonstrated that kin identification and preference lasted through metamorphosis in the frog, *Rana cascadae*. Hence, if kin recognition can carry-over across ontogenetic stages in species with a pelagic larval phase, it could contribute to informed selection of low risk and preferential habitats at settlement (e.g., Vail and McCormick, 2011; Dixon, 2012).

There was a significant difference between the reactions of embryos to kin and non-kin conspecific cues for both species of damselfish, despite the two species having very different life history characteristics. That is, *A. polyacanthus* lacks a pelagic larval stage, has a long egg duration and remain with their parents and siblings for a number of months post-hatching, whereas *A. melanopus* disperse into the pelagic environment as larvae for 15-22 days before settling on the reef (Doherty *et al.*, 1995). This implies kin recognition may be a widespread mechanism in fish species that possess a benthic embryo phase. Additionally, the prevalence of kin recognition in a species seems dependent on the likelihood of encountering relatives in their environment (Carreno *et al.*, 1996; Arnold, 2000). Due to the brooding period of juvenile *A. polyacanthus*, this species spends an extended period of time with their siblings and parents. Furthermore, despite its pelagic larval phase, *A. melanopus*, among other anemonefish and coral reef fish species, often recruit back to their natal reefs; sometimes even settling only metres away from their parents (Jones, 2015). Thus, juvenile *A.*

melanopus are still likely to encounter relatives, meaning kin recognition could be pertinent to coral reef fish species with high levels of self-recruitment.

High sibling association has been found in some other species of reef fishes with pelagic larval stages, which also have lower levels of self-recruitment than my study species. For example, groups of siblings were found in marine gobies (*Coryphopterus personatus*; Selwyn *et al.*, 2016), three-spot *Dascyllus* (*Dascyllus trimaculatus*; Buston *et al.*, 2009) and humbug *Dascyllus* (*D. aruanus*; Bernardi *et al.*, 2012). Selwyn *et al.* (2016) proposed two possible mechanisms through which this could occur. The first is that some larvae remain with their kin throughout the pelagic larval duration, which can be up to a month long in the case of both *Dascyllus* species (Buston *et al.*, 2009; Bernardi *et al.*, 2012). Alternatively, larvae may not be dispersing very far, and instead, remaining in the reef habitat where there is better food availability (Selwyn *et al.*, 2016), which could also explain the high levels of recruitment on natal reefs found in other species (Berumen *et al.*, 2012). These findings, in conjunction with the results of the present study, suggest that kin association may be more important in coral reef fishes than previously realised. However, given the high levels of variability in both pelagic larval durations and dispersal distances in reef ecosystems (Jones *et al.*, 2009; Green *et al.*, 2015), kin association may not be present in, or adaptive for all coral reef fish species. This highlights the importance of research into the prevalence and impact of kin association in the population dynamics of coral reef fish species.

Kin recognition can promote both short and long term advantages. For example, Griffiths *et al.* (2004) found increased foraging rates (long term benefit) and more rapid responses to predatory threats (immediate survival advantage) in brown trout that associated with familiar individuals. Similarly, Schneider and Bilde (2008) confirmed increased growth and foraging efficiency in a spider (*Stegodyphys lineatus*) when associating with kin; and three-spined sticklebacks spent more time with kin members when predators were present (FitzGerald and Morrisette, 1992). The reasoning here

being that by protecting ones kin, individuals are concurrently protecting their gene pool and increasing their inclusive fitness (Hamilton, 1964). Thus, kin recognition and cooperation may decrease the amount of pressure from competition and territorial aggression, allowing for more energy and attention to be focused on foraging and predator avoidance (Gerlach *et al.*, 2007b). In addition, Waldman (1982) suggested that the release of alarm signals is an altruistic act, in that it usually incurs predator-induced mortality, and would only increase a prey individual's inclusive fitness if relatives were nearby to benefit from its release; i.e. kin recognition could be a potential contributing driver for the evolution of alarm cues (for an alternative hypothesis, see Lönnstedt and McCormick, 2015).

The magnitude of response by embryos of both species of damselfish to alarm cues was dependent on the phylogenetic proximity of the cue donor, consistent with studies that examined the relative sensitivities of adults to different donor cues (Cooper *et al.*, 2009; Mitchell *et al.*, 2012). Also, while there seemed to be recognition of cues from the more phylogenetically distant *Chrysiptera cyanea*, neither *A. melanopus* nor *A. polyacanthus* appeared to recognise it as a sufficient indicator of a relevant threat, with the responses not statistically differing from those invoked by the seawater control. Studies on the freshwater gastropod, *Lymnaea stagnalis*, found similar graded responses to alarm cues from heterospecifics of varying phylogenetic distance (Dalesman *et al.*, 2007). In contrast, three-spined sticklebacks showed no discrimination between threats cues sourced from conspecifics or heterospecifics, responding to them equally (FitzGerald and Morrissette, 1992). Dalesman and Rundle (2010) further suggested that the capacity for heterospecific alarm cue recognition is likely dependent on the degree of cohabitation between the species, as well as their relatedness. This is supported by the work of Chivers *et al.* (1995), who showed fathead minnows (*Pimephales promelas*) associate both conspecific and sympatric

heterospecific alarm cues with danger and subsequently avoid areas marked with both cues.

A number of potential mechanisms have been proposed for how organisms are able to distinguish between alarm cues, based on their relevancy and the level of threat they indicate. Kin recognition is widely assumed to be an innate mechanism, but research on fishes suggests there is a potent imprinting and/or learning component (Frommen *et al.*, 2007; Gerlach *et al.*, 2008). Similarly, anemonefish have been shown to possess refined olfactory capabilities allowing them to successfully imprint on specific species of host anemones, which then bias their choice of settlement habitat after their pelagic larval stage (Arvedlund and Nielsen, 1996; Arvedlund *et al.*, 1999 and 2000). Recognition alleles and phenotype matching have also been proposed as potential methods for the recognition of kin via genetic and/or epigenetic mechanisms (Hepper, 1986; Waldman, 1987; Komdeur and Hatchwell, 1999). With regards to the capacity of organisms to differentiate between conspecific and heterospecific cues, Mirza and Chivers (2001b) surmised that cues must either be: (i) identical and contain other chemical components that make them distinguishable; or (ii) similar enough to be recognisable by both species, but vary in their overall composition. Irrespective of the mechanism, threat sensitive reactions to damage-released cues by embryos, demonstrated in both this study, and in **Chapter 5** and Ferrari and Chivers (2010), suggest they hold important survival advantages in early life stages when predation rates are often highest.

Conclusion

My findings not only highlight the importance of innate recognition of kin and relevant risk odours, but also the vital role that threat cues can play in early life stages. Future research directions should include identifying the prevalence of kin recognition and association in juvenile and adult populations of coral reef damselfishes, and determining whether it confers an antipredation survival advantage. It would also be

pertinent to ascertain if kin recognition is present in reef fish species with a lower frequency of self-recruitment. Kin recognition in relation to predator-prey relationships is largely understudied in organisms with complex life cycles due to historically-assumed poor retention of offspring close to the parents. The surprisingly high level of return to natal habitats at the end of the larval phase found in the last two decades (Jones, 2015) underscores the relevance of kin-selection hypotheses to these complex ecosystems, and further research will provide valuable insight into the role kin recognition plays in population and community level dynamics of marine fishes.

Chapter 5: Age matters – embryos

differentially respond to threat cues based on ontogenetic proximity

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5.1 Summary

Predatory risk can vary greatly with both habitat and life history stage. As such, prey species need to be able to recognise and learn relevant predatory threats to avoid wasting energy on unnecessary and energetically costly antipredator behaviours. Damage-released chemical cues provide information for threat-sensitive predator avoidance, allowing prey to adjust their behaviour based on the level of threat. Changes in heart rates of damselfish (*Acanthochromis polyacanthus*) embryos were assessed to see if they were able to differentiate between alarm cues sourced from donors of various ontogenetic stages. Heart rates of embryos increased significantly more in the presence of alarm cues from closer developmental age and relevance. This indicates that damselfish have an innate ability to distinguish among damage-released chemical cues, which could allow them to learn predators most relevant to their life stage and habitat, even before hatching. This information could then be used to avoid predation, select suitable habitat and allocate more energy to fitness-promoting activities, such as foraging and growth in later life stages, all of which would increase their chance of survival.

5.2 Introduction

Predation is a key driver in both population and community dynamics, therefore, identifying relevant predators is an important survival strategy for prey species. Antipredator behaviours involve energetic costs and detract from other fitness promoting behaviours, such as foraging and mating (Houston *et al.*, 1993). While some species have innate recognition of predators, others use sophisticated forms of associative learning with chemical cues to identify risk. In this case, novel odours or shapes are categorised with a level of threat when the perception of the unknown stimulus (potentially a predator) co-occurs with a damage-release chemical cue from a related species (Brown *et al.*, 2011a). These chemical alarm cues are a reliable indicator of threat for aquatic organisms (Smith, 1992). This learned predator information can then be used to reduce the risk of predation without expending energy by reacting to non-predators.

The predators that pose a threat to a prey species change with habitat and life history (Wilbur, 1980; Lönnstedt and McCormick, 2011). This is, in part, due to the gape limitations and size selectivity of predators (Rice *et al.*, 1997; Holmes and McCormick, 2010a; McCoy *et al.*, 2011). For example, a coral reef piscivore, *Pseudochromis fuscus*, uses odours to obtain information concerning the size and body condition of prey, which allows for selection of individuals that do not exceed their maximum gape (Lönnstedt *et al.*, 2012b). Similarly, prey species can obtain information regarding the ontogenetic proximity of donors and concentration of chemical alarm cues to determine the magnitude of risk posed by a predator (Lönnstedt and McCormick, 2011). In systems exhibiting size-selectivity by predators, Slusarczyk *et al.* (2012) found that *Daphnia* can suspend their development and Cecala *et al.* (2015) showed salamanders only display escape behaviours when faced with larger predators; both of which minimised predation risk without overuse of energetically costly antipredator behaviours. The ability to adjust an antipredator response according to the level of risk

has been shown in a range of aquatic and terrestrial taxa (Helfman, 1989; Brown *et al.*, 2009; Ferrari and Chivers, 2009b; Monclús *et al.*, 2009; Chamaillé-Jammes *et al.*, 2013; MacLean and Bonter, 2013). Likewise, some species show variability in developmental timing and/or morphology based on the level of threat experienced in early life stages, which can have life-long consequences (Peckarsky *et al.*, 2001; Peckarsky *et al.*, 2002; Dmitriew, 2011).

While research has shown that juvenile fishes are able to differentiate between cues of different ontogenetic proximities (Mitchell and McCormick, 2013); to date, this threat sensitive recognition has not yet been demonstrated in embryos of any taxa. Recently, eight day old clownfish (*Amphiprion melanopus*) embryos were shown to use innate recognition of conspecific alarm cues to learn predatory risk through association (Atherton and McCormick, 2015). This information can be utilised to avoid predation (Nelson *et al.*, 2013), and select suitable habitats with reduced predatory risk (Dixon, 2012), when settling on a coral reef where predator diversity is very high (Bellwood and Hughes, 2001). Thus, having an innate ability to recognise and utilise cues that are indicative of the level of predatory risk would be an adaptive advantage for potential prey. The aim of my research was to establish whether damselfish embryos are able to distinguish among alarm cues from conspecific donors of different ontogenetic stages. I hypothesised that embryos would react more to cues from a similar life history stage, i.e., heart rate would increase more in the presence of an alarm cue from other embryos than from an adult. Furthermore, I investigated the impact of embryonic exposure to each of the alarm cues on post-hatching juvenile size to determine if threat cues induced differences in growth. My findings indicate a level of behavioural sophistication previously not shown in embryos of any species.

5.3 Methods

a) Study species and animal housing

Acanthochromis polyacanthus (Family: Pomacentridae) is commonly found on the Great Barrier Reef. They reproduce successfully in captivity and their offspring are easy to rear in aquaria as they are the only species of damselfish to lack a pelagic larval stage (Kavanagh, 2000). *A. polyacanthus* produce relatively large eggs for a fish of its size (3.7 – 4.3 mm TL), and embryogenesis (egg development) lasts for eight to fourteen days (Kavanagh, 2000).

Adult breeding pairs of *A. polyacanthus* were housed in well-aerated 70 L tubs, each supplied with recirculated, filtered seawater. The temperature was maintained at 28 ± 1 °C, with a salinity of 35 ppm throughout the breeding season and testing period. Once a clutch of eggs was produced, it was left with its parents until two days before hatching, which was based on known duration of embryogenesis for the breeding pairs during the summer months. Embryos were collected by carefully cutting the adhesive tissue of the egg from the terracotta pot on which it was laid, using a scalpel. Embryos were carefully transferred to a 1 L beaker of seawater from the rearing tank, where they remained until testing. The beaker was kept in a water bath to maintain the temperature at that of the parental tanks and the water was aerated with constant air flow.

b) Stimulus preparation

While the response of fishes to different concentrations of chemical alarm cues has been found to be graded, research has shown that once alarm cues are in high dosage rates, they all elicit strong antipredator behaviours (Brown *et al.*, 2009; Vavrek and Brown, 2009). Hence, I used high concentrations for each type of test cue produced, so that it would signify an ecologically relevant level that simulates mortality in the local environment. Chemical alarm cues from *A. polyacanthus* embryos were produced by

crushing five embryos in a petri dish and rinsing them with 5 ml of seawater. The solution was then filtered through filter paper to remove any particulate matter; the resultant 5 ml odour-infused seawater provided cues for five test trials. Chemical alarm cues from juveniles (15 ± 2 mm TL; 1 month old) were produced by euthanising juveniles with a single blow to the head and superficially cutting along each side of the fish three times. Each fish was then rinsed with 5 ml of seawater, which was then filtered, resulting in 5 ml of cue, which was enough for five trials. Cues from adult *A. polyacanthus* (65 – 70 mm TL) and heterospecifics (*Xiphophorus hellerii*, 60 – 65 mm TL) were made using the same method as the juvenile cues, except six and ten cuts were made both sides of each adult and heterospecific fish, respectively, and then both types of donor fish were rinsed with 10 ml of seawater, producing 10 trial cues. *X. hellerii* (swordtails) were selected as a heterospecific control as they are known to release alarm cues, but are also phylogenetically distant from *A. polyacanthus* (Larson and McCormick, 2005). All cues were produced in small batches continuously throughout the experimental trials to prevent degradation.

c) Experimental procedure

Neurophysiological responses, such as changes in heart and ventilation rates, have been shown to be associated with predator recognition and often accompany antipredator behaviours, such as fleeing (Smith and Johnson, 1984; Höjesjö *et al.*, 1999; Barreto *et al.*, 2003; Oulton *et al.*, 2013). As such, change in embryo heart rate was measured as a behavioural proxy in response to the presence of one of five different chemical stimuli. The five cues used were: seawater control, or alarm cues prepared from embryos, juveniles or adult conspecifics (*A. polyacanthus*), or freshwater heterospecifics (*X. hellerii*). Single *A. polyacanthus* embryos were placed in a small white dish containing 10 ml of seawater, under a dissecting microscope with a cold light. They were allowed to acclimatise for 2 min, after which their heart rate was recorded for 30 s. One ml of cue was then slowly injected into the 10 ml container, and

heart rate was immediately recorded for a further 30 s. Fifteen embryos from each clutch were tested against one of the five cues, and embryos were sourced from four different clutches from three separate breeding pairs.

d) Juvenile development

On completion of the heart rate trials, the fifteen embryos tested for each cue were placed into separate, 9 L rearing tanks (i.e., 1 tank for embryos from each of the 5 test cues, per clutch). An additional 45 embryos were treated with the same concentration (1 ml of cue per 10ml seawater, for each embryo) one of the five test cues (seawater, or embryo, juvenile, adult or heterospecific alarm cues), resulting in a total of 60 treated embryos per cue. Furthermore, 60 untreated/untested embryos from the same clutch were placed in a sixth rearing tank; this represented an undisturbed control. After hatching, the *A. polyacanthus* juveniles were fed freshly hatched *Artemia* nauplii (around 250 individuals per ml), twice daily, at a concentration of 0.5 ml per individual, per tank for the first seven days and then 1 ml per individual, per tank from then on. On day 21 post-hatching, each juvenile was individually captured in a small zip-lock bag containing a small amount of water, placed on a 5x5 mm grid, and photographed; the juvenile was then returned to the tank. Using ImageJ, four morphological measurements (mm) were obtained from each juvenile's photograph; these included standard length, body depth, caudal peduncle depth and eye diameter (Figure 5.1).

e) Statistical analyses

Change in heart rate was used as the raw data for analyses, and residual analyses showed that the data met the assumptions of ANOVA. The ANOVA model tested cue and clutch, and the interactions between them, as fixed factors. A Tukey's HSD post hoc test was used to determine the nature of significant differences found by ANOVA.

A two-factor MANOVA was conducted to test Cue (fixed) and Clutch (random), and the interaction between them, in relation to the four morphological measurements (standard length, body depth, peduncle depth and eye diameter). The raw data violated the MANOVA assumption of homogeneity of the variance-covariance matrix (Box M < 0.001), so a Pillai's trace was used as the test statistic because it is robust to violations of this assumption.

e) Ethics statement

This research was approved by the James Cook University Animal Ethics Committee under the approval permit A1871.

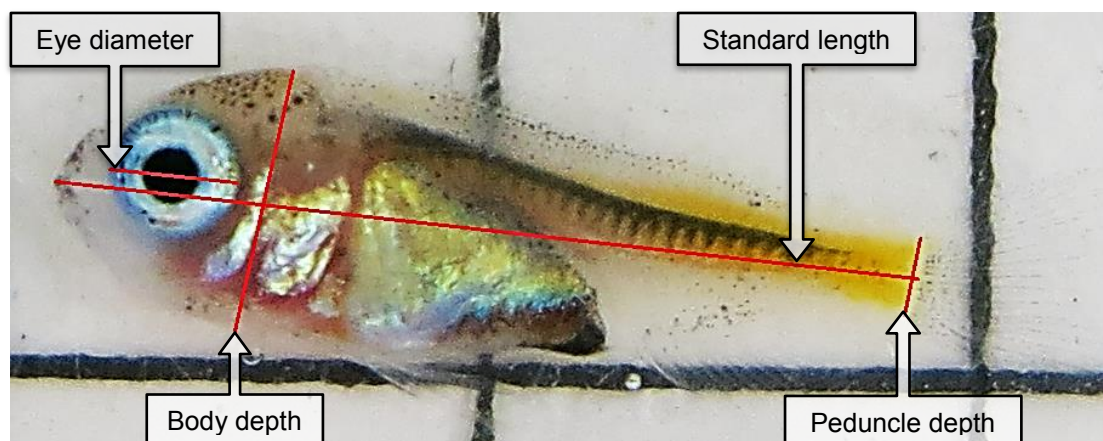


Figure 5.1: Four morphological measurements (mm) taken from each three week old juvenile *A. polyacanthus*; standard length, body depth, peduncle depth and eye diameter.

5.4 Results

a) Changes in embryo heart rates

There was a significant difference in the change in embryo heart rate induced by the five chemical cues ($F_{4,330} = 47.286$, $P < 0.0001$; Figure 5.2). The reaction did not differ between clutches and was consistent among clutches (non-significant clutch and cue interaction; Table 5.1). The introduction of an embryo alarm cue induced a large increase in heart rate, which differed significantly from all other cues (+12.3%; Tukey's HSD: $P < 0.0001$; Figure 5.2).

The introduction of the juvenile cue, resulted in a similar but reduced increase in embryo heart rate of +7.1%, which also differed significantly from all other cues used (Tukey's HSD: $P < 0.0001$). The cue sourced from adult *A. polyacanthus* caused a significantly smaller increase in heart rate compared to embryo and juvenile conspecific donors (+3.0%; Tukey's HSD: $P < 0.0001$). Both the seawater and the heterospecific control cues caused negligible increases in heart rate (+0.1% and +0.22%, respectively), which differed significantly from the reactions induced by cues from the early ontogenetic stage conspecifics (Tukey's HSD: $P < 0.0001$; Figure 5.2).

Table 5.1: A two-factor ANOVA comparing the change in heart rates induced by alarm cues from donors of varying ontogenetic proximity, and the clutch from which the embryos were obtained.

Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Clutch	2	1.649	0.054	0.9476
Cue	4	1448.107	47.286	< 0.0001
Clutch*Cue	8	11.207	0.366	0.9380
Residual	330	30.625		

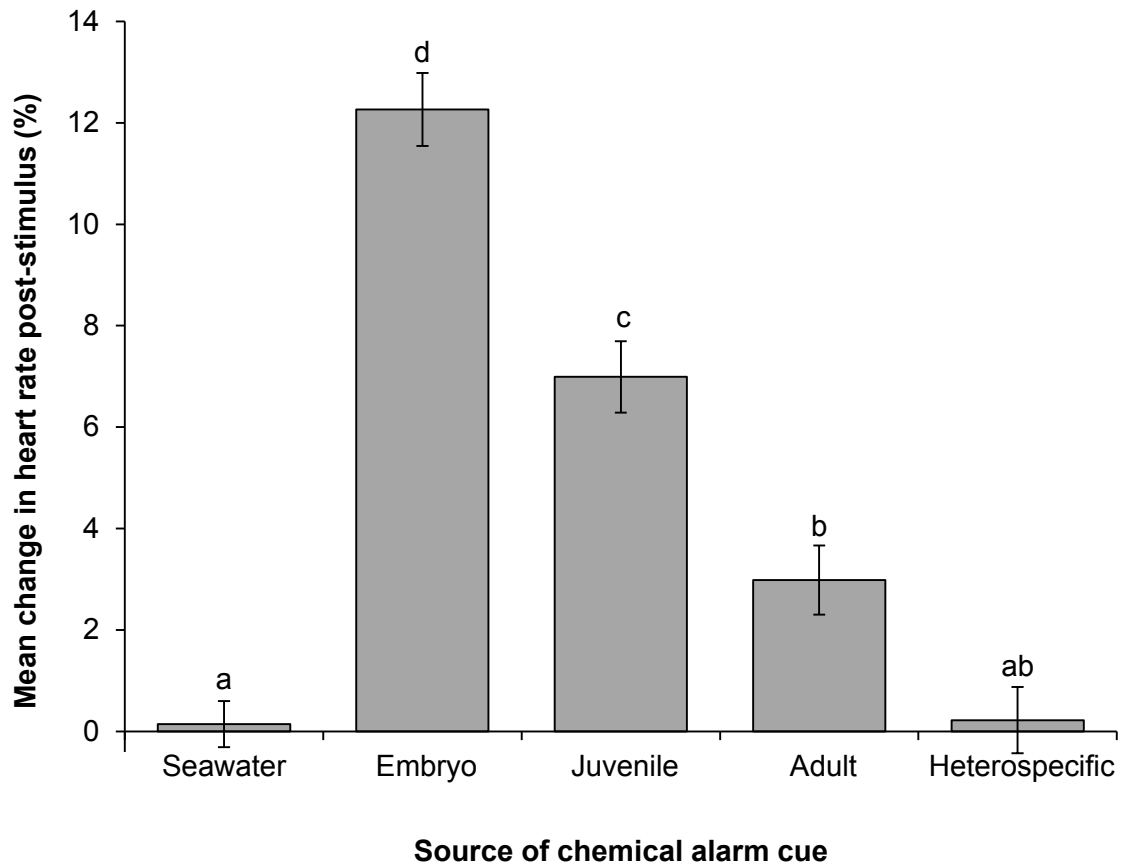


Figure 5.2: Reaction from pre-stimulus conditions of *Acanthochromis polyacanthus* embryos to seawater (control) or seawater containing one of four damage-released cues originating from: conspecific embryos, juveniles, or adults, or a heterospecific (*Xiphophorus hellerii*). Letters represent Tukey's HSD groupings of means ($N = 75$ for all test cues, except for 'Heterospecific', for which $N = 45$).

b) Juvenile growth

Juvenile size at 21 days post-hatching did not differ significantly in relation to the cue received as an embryo for any of the morphological measurements (Table 5.2; Figure 5.3. However, Clutch and the interaction between Clutch and Cue were both significant (Table 5.2), meaning clutch term generated the majority of the variance in the MANOVA model (Figures 5.4a and 5.4b).

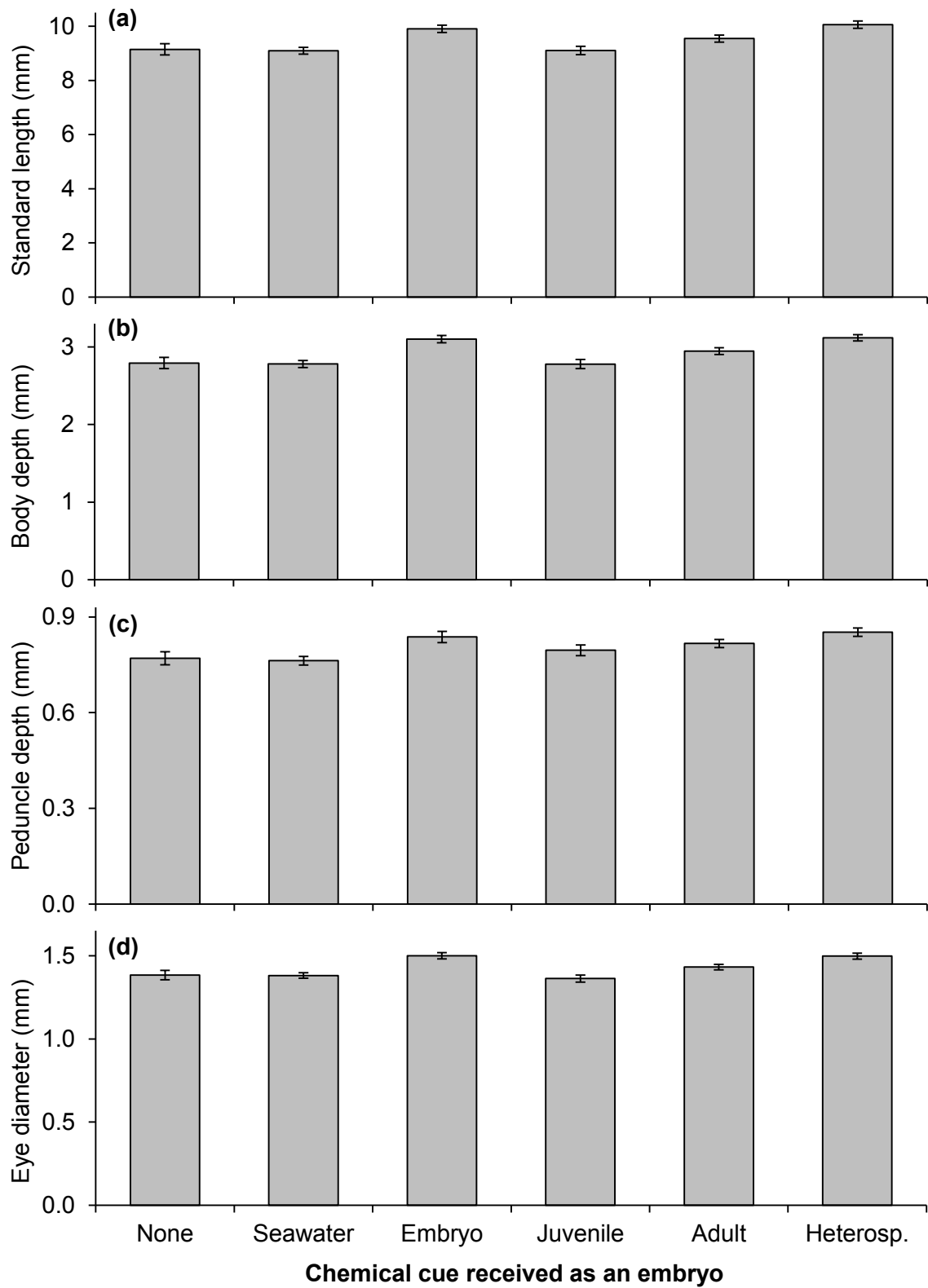


Figure 5.3: Mean juvenile size (mm \pm SE) across four morphological measurements: **(a)** standard length, **(b)** body depth, **(c)** peduncle depth, or **(d)** eye diameter; assessed on day 21 after hatching. As embryos, these juveniles were exposed to no cue, or one of five chemical cues (seawater, or an embryo, juvenile, adult or heterospecific alarm cue).

Table 5.2: A two-factor MANOVA testing Cue (fixed), Clutch (random) and the interaction between them, where four separate morphological measurements were included as the dependent variables.

Morphological measurement	Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Standard length	Clutch	2	150.138	32.849	<0.0001
	Cue	5	1.505	0.332	0.8823
	Clutch x Cue	10	4.758	22.463	<0.0001
	Residual	324	0.212		
Body depth	Clutch	2	17.393	31.039	<0.0001
	Cue	5	0.246	0.443	0.8094
	Clutch x Cue	10	0.583	20.771	<0.0001
	Residual	324	0.028		
Peduncle depth	Clutch	2	2.586	40.102	<0.0001
	Cue	5	0.033	0.515	0.7598
	Clutch x Cue	10	0.067	15.024	<0.0001
	Residual	324	0.005		
Eye diameter	Clutch	2	1.381	29.493	<0.0001
	Cue	5	0.019	0.401	0.8375
	Clutch x Cue	10	0.059	10.487	<0.0001
	Residual	324	0.005		

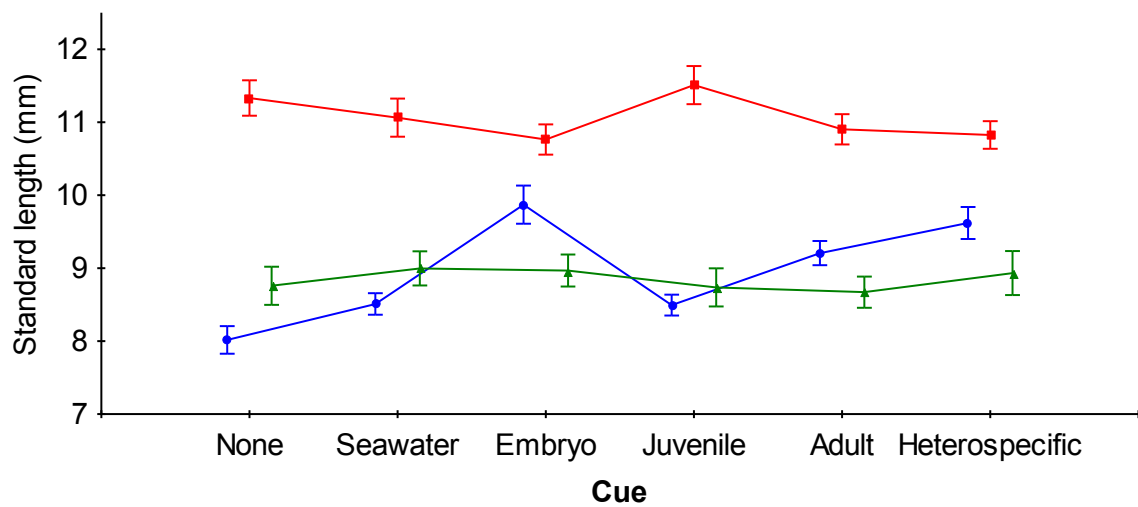


Figure 5.4a: Interaction plot for Cue and Clutch for the standard length (mm \pm SE) of juveniles. Each line (red, green and blue) represents each of the three clutches.

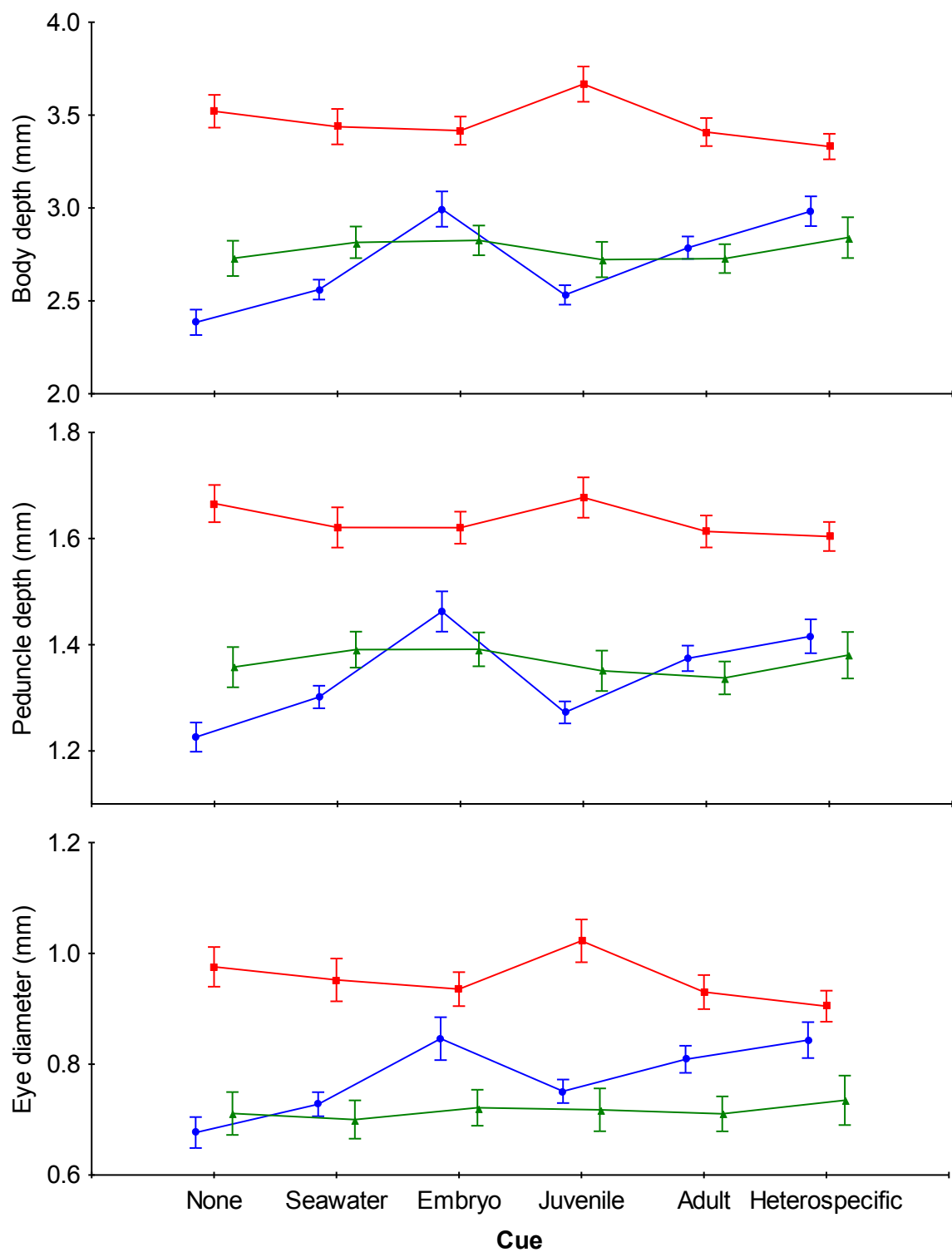


Figure 5.4b: Interaction plots for Cue and Clutch for three of the four morphological measurements; body depth, peduncle depth and eye diameter (mm \pm SE). Each line (red, blue and green) represents one of the three clutches, respectively.

5.5 Discussion

Embryos of *Acanthochromis polyacanthus* were found to have a sophisticated mechanism of discriminating among olfactory cues, reacting more to cues created from conspecifics of a similar developmental stage. This is the first time that such a finely graded response, and recognition of ontogenetic proximity, has been documented in an embryo of any species. This supports other research on fishes which indicate that juveniles and adults can differentiate between cues based on life history relevancy (Lönnerstedt and McCormick, 2011), using only the most pertinent cues to associatively learn predatory threats (Mitchell and McCormick, 2013). Belden *et al.* (2000) also demonstrated that juvenile toads (*Bufo boreas*) use diet cues from predatory snakes to avoid habitats where other juvenile conspecifics had been consumed, but not younger, conspecific larvae.

Many species with complex life histories undergo shifts in diet and habitat with ontogenetic changes (Werner and Gilliam, 1984). Such transitions could be the reason that detectable differences occur between cues from conspecifics of different ages/sizes. Mirza and Chivers (2002) proposed that cues from different ontogenetic stages could have: (i) the same basic chemical composition with additional variations; or (ii) similar and recognisable, but not identical compositions; both of which could generate the differential responses observed in my study.

As *A. polyacanthus* lacks a pelagic larval stage (Kavanagh, 2000), the predators that pose a threat to embryos (e.g., yellow dottedback, *Pseudochromis fuscus*), are also likely to prey on juveniles (Feeney *et al.*, 2012). The life history of *A. polyacanthus* is quite unique for a reef fish, but is very similar to that of a number of amphibian species in that embryos hatch into their parental environment (Wilbur, 1980). In addition, self-recruitment levels can be quite high in some species of coral reef fishes with pelagic larval stages (Jones *et al.*, 2015); so, predators in the embryonic environments would

also likely be relevant to settlement stage juveniles. Thus, embryonic recognition of cues signifying predatory threats could increase the chance of survival in later life stages; a phenomenon known as a carry-over effect. For example, exposing embryos to predator odours reduced their level of activity (a common antipredator defence) at the larval stage, in fishes (Nelson *et al.*, 2013) and amphibians (Mathis *et al.*, 2008). However, in the present study, the introduction of a relevant threat cue only induced tachycardia, which was not accompanied with a predator avoidance response, such as premature hatching as seen in Cohen *et al.* (2016), but not this study (qualitative researcher observation). As such, the juvenile alarm cue would likely be the most relevant cue for the embryos, because as post-hatching juveniles, they would be able to avoid predation by performing antipredator behaviours. Hence, our results could be a demonstration of this species' olfactory system developing in preparation for the 'hard-wired' ability to discriminate between cues of varying ontogenetic relevance and act according to the level of threat they infer (Mitchell and McCormick, 2013).

Variations in cue concentration have resulted in threat sensitive learning in wood frog embryos, which were also able to apply acquired predator knowledge to other likely threats (Ferrari and Chivers, 2009a). Additionally, rainbowfish embryos have shown threat awareness before hatching, responding to native predator cues with an increase in heart rate and a slightly delayed hatching time (Oulton *et al.*, 2013). Heart rate, among other neurophysiological responses, has been used in conjunction with antipredator behaviours to assess predator recognition in terrestrial and aquatic taxa (Smith and Johnson, 1984; Johnsson *et al.*, 2001). Ydenberg and Dill (1986) suggested that changes in heart rate can provide insight into an individual's awareness of a predator, even before any avoidance behaviours are observed. Indeed, an increase in heart rate in response to a threat cue in an immobile embryo may not seem like it serves an adaptive purpose, yet research on crucian carp (*Carassius carassius*) showed a similar initial tachycardic response to predator presence. Prolonged predator

exposure, however, resulted in a decrease in overall resting heart rate, and greater frequency of sheltering behaviour, increasing energy allocation for growth (Holopainen *et al.*, 1997).

The type and relevancy of the chemical cues that *A. polyacanthus* were exposed to as embryos had no effect on the size of juveniles at three weeks. There are numerous examples of studies investigating predator induced phenotypic plasticity (i.e., changes in growth as a result of developing in a risky environment; reviewed in Bernard, 2004). While some research contradicts our findings and demonstrated a significant effect of embryonic threat cue exposure on juvenile development (Orizaola and Braña, 2005), this appears to be species and/or context dependent, rather than a widespread mechanism in nature. In the present study, all juveniles were all reared in the absence of threat cues, which could explain the observed similarity in juvenile size across treatments. Concurrently, Reylea (2003) and Orizola *et al.* (2012) found that predator induced morphologies could be reversed if the predator was removed from the environment.

Conclusion

Innate discrimination between cue donor ontogeny at the embryonic life stage suggests that alarm cue relevancy could also play a vital role in determining risk, especially in environments with high predator diversity. These findings corroborate the threat-sensitive predator avoidance hypothesis (Helfman, 1989), and demonstrate the intricate and complex interactions involved in promoting survival through antipredator behaviours. Environmental awareness of embryos is a relatively new field of ecological research, especially with regards to knowledge acquisition in embryonic fishes. Further study is required to determine the impact these olfactory capabilities have on the behaviour and survival of later life stages.

Chapter 6: General Discussion

Early experience can be vital to the success and survival of young and developing individuals. The ability of parents to impart predator information to their progeny can give offspring a head start in life. This inherited information can then be built on by embryos using their own olfactory capabilities, allowing them to hatch with a well-developed early awareness of the present threats in their surrounding environment. This thesis demonstrates the first examples of predator-induced parental effects, embryonic learning, and the ability of embryos to distinguish between cues in a threat sensitive manner, in marine fish species. Due to the highly diverse nature of coral reefs, prey are at risk of predation from a wide range of species, which can vary greatly in both space and time. Thus, the importance of a good start with a prior knowledge of local threats, and the ability to rapidly learn new threats, can mean the difference between surviving to reproduction and not.

Transgenerational predator recognition

The effect of increased predation risk on offspring via parental effects has been studied in a range of taxa. However, **Chapter 2** of this thesis provides the first example of parents conveying a specific predator identity to their offspring via a non-genetic mechanism. While the research did not identify the mechanism through which this transfer occurred, the most likely methods are epigenetic and/or hormonal pathways. Mommer and Bell (2014) investigated differences in gene expression in three-spined stickleback embryos, comparing offspring from parents in environments either with or without predators. Their results showed large differences in whether genes were up- or down-regulated between parental treatments, with genes involved in metabolic control being some of those affected. This was suggested to be one of the controlling factors

for the embryos of predator-exposed parents being larger, indicating epigenetic/molecular mechanisms for predator-induced parental effects. Alternatively, Coslovsky *et al.*'s (2012) study on the great tit identified lower levels of testosterone in the egg yolk of predator exposed parents compared to controls. This hormonal modification by parents in risky environments induced developmental differences in their progeny, resulting in smaller offspring with longer wings at maturity (Coslovsky and Richner, 2011a).

Predator-induced parental effects can carry numerous adaptive advantages for offspring. Progeny produced by parents in high risk environments exhibit a higher propensity for antipredator behaviours, such as tighter shoaling behaviour in sticklebacks (Giesing *et al.*, 2011), immobility in crickets (Storm and Lima, 2010), and higher sensitivity to predator cues in skinks (Shine and Downes, 1999). Additionally, morphology can be altered by predator-induced parental effects, resulting in phenotypic traits that promote offspring survival. For instance, smaller birds with larger wings (Coslovsky and Richner, 2011a), longer tails in lizards (Bestion *et al.*, 2014), and a higher proportion of winged offspring in aphids (Mondor *et al.*, 2005). In systems with high species diversity, conveyance of *specific* predator information from parents to can also help offspring to identify other phylogenetically similar predators (Ferrari *et al.*, 2007; Mitchell *et al.*, 2013). Thus, through transgenerational predator recognition, parents could be endowing their progeny with the knowledge of not only one, but a suite of relevant predator identities.

In contrast, predator-induced parental effects can also carry maladaptive consequences for offspring. Research on threespined sticklebacks found diminished learning capabilities (Roche *et al.*, 2012) and a decrease in antipredator behaviours and survival (McGhee *et al.*, 2012) in offspring of predator-exposed mothers. Furthermore, Coslovsky and Richner (2012) found great tit chicks fledged a day later if there was a mismatch between the level of predatory risk in the parental and offspring

environments. However, there is the potential to compensate for any negative consequences caused by predator-induced parental effects through offspring flexibility in growth (Gagliano and McCormick, 2007), or by becoming more dependent on social cues and learning. For example, Feng *et al.* (2015) found offspring of mother sticklebacks exposed to predators were quicker to copy a trained individual than offspring from control mothers; this reliance on social learning allowed them to overcome any maladaptive consequences of maternal stress. Hence, although transgenerational predator recognition may provide an early warning of threats, it appears that individual experience and current environmental conditions play a vital role in promoting offspring survival.

While **Chapter 2** demonstrates that parents can help to prepare their offspring for an environment containing predatory threats, **Chapters 3 – 5** demonstrate that damselfish are also able to obtain information about their environment using risk cues, during embryogenesis. Combined, this transgenerational predator recognition and embryonic learning and awareness could provide early life stage prey species with refined recognition of threats and an effective means for threat-sensitive predator identification after hatching.

Embryonic olfactory capabilities

Damselfishes develop at different rates and with embryogenesis lasting from a couple of days to a couple of weeks in some species (Arvedlund *et al.*, 2000; Kavanagh and Alford, 2003). Prior to hatching, the olfactory apparatus in most damselfish embryos develops to a point where they are capable of imprinting on natal odours (Arvedlund *et al.*, 2000), detecting threat cues (**Chapters 4 and 5**), and learning through association (**Chapter 3**). Chemosensory learning has been demonstrated in embryos in frogs and salamanders (Mathis *et al.*, 2008), cichlids (Nelson *et al.*, 2013) and cuttlefish (Romagny *et al.*, 2012). Early learning of threats by embryonic-stage prey species can

enhance survival chances through increased predator recognition and antipredator responses after hatching (Ferrari and Chivers, 2013). Coral reef fishes are often assumed to be naïve to predators at settlement. Yet, research suggests that anemonefishes may be able to use their advanced olfactory abilities to imprint on host anemones (Arvedlund *et al.*, 2000), while simultaneously learning predatory threats (**Chapter 3**). Combined, this can provide larvae with a sophisticated means to orientate back to their natal reef (Gerlach *et al.*, 2007a; Dixon *et al.*, 2008; Leis *et al.*, 2011) and select a safe and suitable habitat at settlement (Vail and McCormick, 2011; Dixon, 2012).

Using associative learning, the level of risk a prey species assigns to a predator can depend on the concentration and/or relevance of the cue that the alarm cue is paired with. The energetic trade-off between antipredator behaviours and other fitness-promoting activities (e.g., growth, foraging and reproduction; Houston *et al.*, 1993; Werner and Anholt, 1993) suggests that reacting in a threat-sensitive manner to risk cues can allow individuals to conserve energy (Helfman, 1989; Brown *et al.*, 2006; Vavrek and Brown, 2009). Using the level of risk denoted by an alarm cue (e.g., increased concentration) to label a predator as posing a greater threat, can further allow prey species to avoid predation while not unnecessarily carrying out costly antipredator behaviours (Ferrari *et al.*, 2005 and 2006; Brown *et al.*, 2011a). Mitchell and McCormick (2013) demonstrated that juvenile coral reef fish only used juvenile alarm cues to associatively learn a predatory threat, and did not deem an alarm cue sourced from a conspecific adult as a good indicator of a relevant threat, thus failing to use it to learn a threat. Being selective of which cues are used for learning can allow prey to match their response to the level of threat posed by a predator, in accordance with the threat-sensitivity hypothesis (Helfman, 1989).

Combined, the research in **Chapters 4 and 5** highlights the importance of being able to not just detect a wide range of alarm cues, but also possess the ability to determine

their relevance. For example, **Chapter 4** found two species of damselfish with different life histories (*Acanthochromis polyacanthus* and *Amphiprion melanopus*) could distinguish between cues based on the phylogenetic relatedness of the fish from which the cue was sourced. Additionally, **Chapter 5** demonstrated threat-sensitive responses to cues sourced from different ontogenetic stage conspecifics. Therefore, coral reef fish embryos appear to differentiate between alarm cues based on a relevancy spectrum, with introduction of the most relevant cues eliciting a greater increase in heart rate response (Figure 6.1). For embryonic-stage damselfish, the most relevant cues are those emitted by conspecifics of a similar life history stage. However, in the absence of an immediate predator escape response in embryos, such as premature hatching (Warkentin, 1995; Cohen *et al.*, 2016), the adaptive advantage of responding more to an embryo alarm cue is unclear. One would expect that, for an embryo, a conspecific juvenile alarm cue would be more pertinent, so when the embryo is mobile post-hatching, they are able to quickly recognise and avoid relevant predators. Ontogenetic differences detected in cues can be a good predictor of predatory relevance, as gape-limitation means predators tend to target prey of a similar size, irrespective of species (St. John, 1999). Moreover, recognition of closely related heterospecific cues can be important in coral reef habitats where generalist predators and coexistence in complex habitats are common (Goldman and Talbot, 1976; Waldner and Robertson, 1980). Coral reefs are biodiversity hotspots, so being able to eavesdrop and distinguish between heterospecific cues as well as conspecific cues can provide prey species with valuable insight into relevant threats in the environment.

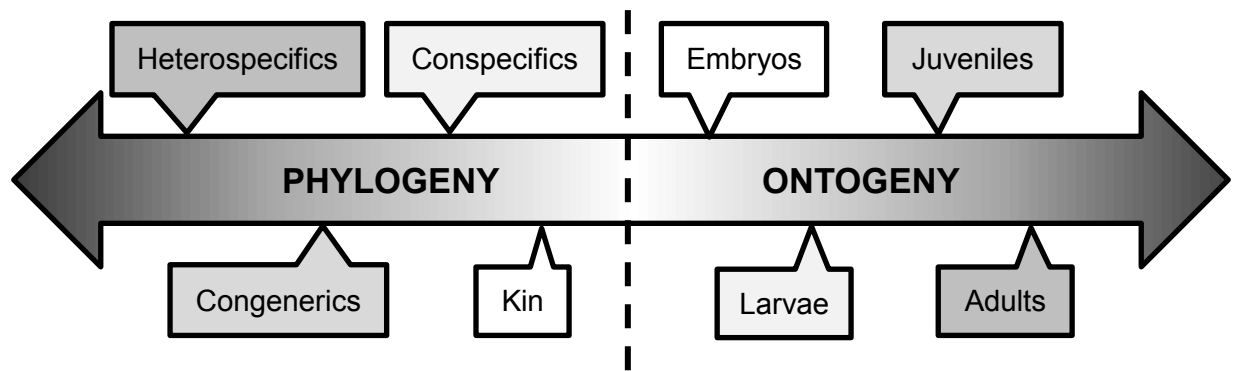


Figure 6.1: Alarm cue relevancy spectrum for an embryo stage individual (indicated by dashed line). The further from the dashed line, the less relevant the cue.

Kin recognition forms another potentially important mechanism for survival in early life-stage prey species, which is largely understudied in coral reef ecosystems and marine fishes in general. **Chapter 4** demonstrates that two species of damselfish, and potentially even all demersally spawned reef fishes, possess olfactory recognition of kin using damage-released alarm cues. Further research is required to determine the exact adaptive significance and the purpose(s) for this olfactory discrimination. However, based on the findings of other research, it is likely that kin recognition exists in these species due to the selective advantages of cooperating with relatives, which can boost individual fitness as well as inclusive fitness. For example, improved foraging efficiency, increased growth and having greater awareness of predators have all been shown in species that associate with related individuals (Griffiths *et al.*, 2004; Schneider and Bilde, 2008; Thünken *et al.*, 2016). This is likely due to reduced competition pressure arising from cooperating with kin (Gerlach *et al.* 2007b), which concurrently boosts inclusive fitness by protecting the collective gene pool (Hamilton, 1964). While this is the first time that olfactory kin recognition has been demonstrated in coral reef fish species, genetic analyses of fish populations have found sibling association in a number of other reef fishes (Planes *et al.*, 2002; Selkoe *et al.*, 2006; Buston *et al.*, 2009; Bernardi, *et al.*, 2012; Selwyn *et al.*, 2016). Hence, kin selection

may be more prevalent and important in reef fish population dynamics than has been previously realised.

Implications of early information on key life history stages

Early knowledge of predatory threats in embryos (obtained through parental effects [Chapter 2] or embryonic learning [Chapter 3]) could increase early life stage prey species' chances of survival in a variety of ways. Although it was not identified in my research, other studies have demonstrated early/delayed hatching in aquatic taxa when exposed to an immediate predatory threat (Warkentin, 2000, Chivers *et al.*, 2001; Warkentin, 2005). However, when there is a constant threat present during embryogenesis, prey can respond with adaptations that will be more likely to benefit them in the long term in high risk environments. For instance, predator presence can cause induced defences (Laforsch and Tollrian, 2004), morphological and developmental differences (Mandrillon and Saglio, 2007; Mourabit *et al.*, 2010), and/or modifications in the propensity for antipredator behaviours (Mathis *et al.*, 2008; Nelson *et al.*, 2013). These mechanisms can all promote survival post-hatching in early life stages when the risk of predation is highest, in ecosystems with numerous and diverse predatory threats.

The high levels of self-recruitment seen in some species coral reef fishes with pelagic larval durations (Berumen *et al.*, 2012; Jones, 2015) and the low levels of dispersal seen in *Acanthochromis polyacanthus* (Miller-Sims *et al.*, 2008) mean that local predator identities acquired/learned before hatching are likely to still be relevant to prey species as larvae and juveniles. Prior knowledge of predatory threats increases the chance of an individual avoiding predation through early recognition (Lönnerstedt *et al.*, 2012a). Recruiting back to their natal reef also increases the chance of juveniles encountering genetic relatives, rendering kin recognition pertinent to coral reef damselfish. Associating with kin can allow prey species to focus more of their attention

on foraging and avoiding predation, thus increasing their chance of surviving to reproduction and contributing to the gene pool (**Chapter 4**). In contrast, Bestion *et al.* (2014) found greater levels of dispersal in juvenile lizards whose parents had been exposed to predators; an adaptive maternal effect allowing offspring to avoid predation through informed habitat choice. Transgenerational predator recognition, along with the capacity for embryos to learn predator identities can allow juveniles to select a suitable habitat based on the presence/absence of threats (Vail and McCormick, 2011; Dixon, 2012).

After choosing a suitable settlement habitat, juveniles may need to update their current knowledge of predatory threats to include new species, or consolidate the level of threat posed by the predators they have already catalogued. **Chapters 2 and 3** demonstrate that innate recognition (i.e., a response to a threat cue which the individual itself has not previously experienced) of a novel predator can be upregulated by both parental and own experience. In order to avoid predation, prey species need to continually update the information they have regarding predatory threats in their current environment (Mitchell *et al.*, 2011a), and do so using cues from donors of a similar life stage to learn which predators are relevant at a particular ontogenetic stage (**Chapter 5**). Similarly, without reinforcement with chemical alarm cues, prey can eventually fail to recognise a predator cue as being indicative of a threat (Ferrari *et al.*, 2010c; Chivers and Ferrari, 2013). Using this range of methods for ascertaining predatory threats, coral reef damselfish can increase their chance of avoiding predator-induced mortality, survive to reproduction, and begin the whole cycle again by transferring their knowledge of relevant predator identities to offspring via parental effects (**Chapter 2**).

Future research directions

My research has provided the foundation for many future investigations into the importance predator-induced parental effects and embryonic detection and associative

learning of threats in coral reef fishes. However, as with most investigations into a previously unstudied field of research, it poses more questions than it answers. Now I have demonstrated that transgenerational predator recognition occurs in a coral reef fish species (**Chapter 2**), studies should be conducted to identify the mechanism for this transfer of information and the impact it has on post-hatching performance in offspring. Regarding the observed embryonic associative learning of predatory threats (**Chapter 3**) and threat-sensitive detection of chemical cues (**Chapters 4 and 5**), key future research directions should include: determining the adaptive/maladaptive consequences of embryonic tachycardic responses to threat cues; identifying the developmental, behavioural and survival implications of pre-hatching recognition of threat cues; and evaluating the extent to which these processes occur in other coral reef fish species with varying levels of dispersal, self-recruitment, parental care and sibling association. Overall, importance and prevalence of parental and embryonic predator exposure on offspring survival across coral reef fish species could provide important insight into some of the currently unexplained mechanisms driving population dynamic processes in reef fishes.

The bigger picture

Predation is a key driver of population dynamics (Lima and Dill, 1990; Petorelli *et al.*, 2011). Coral reefs have been described as a ‘predation gauntlet’ for juvenile fishes (Almany and Webster, 2006), partly due to the high diversity of predators that fish at the end of their larval stage are likely to encounter, but also because early life stage fishes suffer very high predator-induced mortality rates. Hence, removal of predatory species that are highly sought after by both recreational and commercial fishing can have cascading top down effects on the rest of the ecosystem. There is an inverse relationship between the abundance of predatory and prey species in that as the number of predators decreases due to fishing, species’ abundances in lower trophic levels of the food web increase (Caley, 1993; Graham *et al.*, 2003; Stallings, 2008). In

order to establish a solid framework for future conservation management strategies, we have to first understand the ecology of a system and the drivers of population and community dynamics. With climate change and fishing pressure changing the face of coral reefs worldwide, such information could help formulate informed predictions about the adaptive capacity of prey species in the future. While this research only scratches the surface in terms of the effect(s) of predator presence on early life history of coral reef fishes, it provides valuable insight into the complex and refined mechanisms fishes possess for recognising and learning predatory threats.

Concluding remarks

This thesis demonstrates that embryonic damselfishes have sophisticated olfactory capabilities, with the capacity to not only learn predatory threats through association as soon as their olfactory system has developed, but they can also differentiate between cues based on their phylogenetic and ontogenetic proximity. Combined, these chemosensory mechanisms can enable embryos to catalogue a range of threat cues before they have even hatched out of the egg. In addition, parents are able to transfer information regarding specific predator identities to their offspring via parental effects. By upregulating what appears to be an innate recognition of predator cues, using parental and/or an embryo's own experience, early life stage individuals can respond in a threat-sensitive manner to predatory threats and use this information to select a safe habitat at settlement. Furthermore, my research demonstrates that embryonic damselfish clearly recognise their kin, which combined with the high levels of self-recruitment and sibling associated seen in some species of coral reef fishes, can allow individuals to work cooperatively and/or increase their inclusive fitness.

By starting to classify and catalogue threats from the earliest possible stage in their life cycle (gametogenesis – gamete production by parents, and embryogenesis – egg development), prey species could prevent unnecessary energy expenditure while

avoiding predation, and instead select safe habitats for settlement and allocate more energy to foraging, growth and reproduction. Overall, my thesis highlights the importance of parental and embryonic predator environments to offspring risk assessment, and the vital role early recognition of threats plays in promoting survival in the most vulnerable life stages.

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Appendices

Appendix 1: Chapter 2 pilot trial

A pilot study was undertaken to show whether it is possible to use cues that had been frozen in liquid nitrogen as a viable replacement for freshly collected cues, using the example of the dottyback, *Pseudochromis fuscus*. The fresh dottyback cue induced a 5.81% increase in heart rate, compared to the 5.51% increase caused by the frozen and defrosted cue; a two-factor ANOVA showed there to be no significant difference between the two (Table A2.1). Freezing and storing cues prior to test trials reduced the amount of handling stress placed on all three species of cue donor fishes.

Table A2.1: A two-factor ANOVA comparing the change in heart rate induced by the type of dottyback (*Pseudochromis fuscus*) cue used (fresh or frozen), and the clutch from which the *Acanthochromis polyacanthus* embryos were sourced.

Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Pair	2	16.549	0.721	0.4890
Cue type	1	2.027	0.088	0.7670
Pair x Cue type	2	0.551	0.024	0.9763
Residual	84	22.939		

Appendix 2: Chapter 4 pilot trial

A pilot trial was conducted to determine if it was possible to freeze embryos for later use as cue donors in embryo behaviour trials. This would allow for a wider range of samples to be tested, such as testing recently produced embryos with clutches previously produced by the same breeding pair.

The experiment used *Amphiprion melanopus* and *Acanthochromis polyacanthus* embryos to compare the reactions to cues created using either fresh embryos, embryos frozen in liquid nitrogen, or a seawater control. Embryos were sourced from clutches produced by three separate breeding pairs, for each species, with fifteen embryos from each being tested against one of the three cues. A linear mixed-effects ANOVA model tested cue type (fresh or frozen) and test species as fixed factors, but also included clutch as a random factor.

The results demonstrated that chemical alarm cues created from frozen (and defrosted) embryos produced a very similar level of response to fresh alarm cues (Tukey's HSD: $P = 0.59$; Figure A4.1 and Table A4.1). Hence, it was deemed acceptable to use alarm cues produced from frozen embryos.

Table A4.1: A two-factor fixed ANOVA comparing the change in heart rate induced by alarm cues produced from fresh or frozen and defrosted embryos, which was crossed with the species that was tested.

Effect	<i>df</i>	MS	<i>F</i>	<i>P</i>
Cue	2	83.742	189.737	< 0.0001
Species	1	0.105	0.239	0.6255
Cue * Species	2	0.086	0.195	0.8232
Residual	264	0.441		

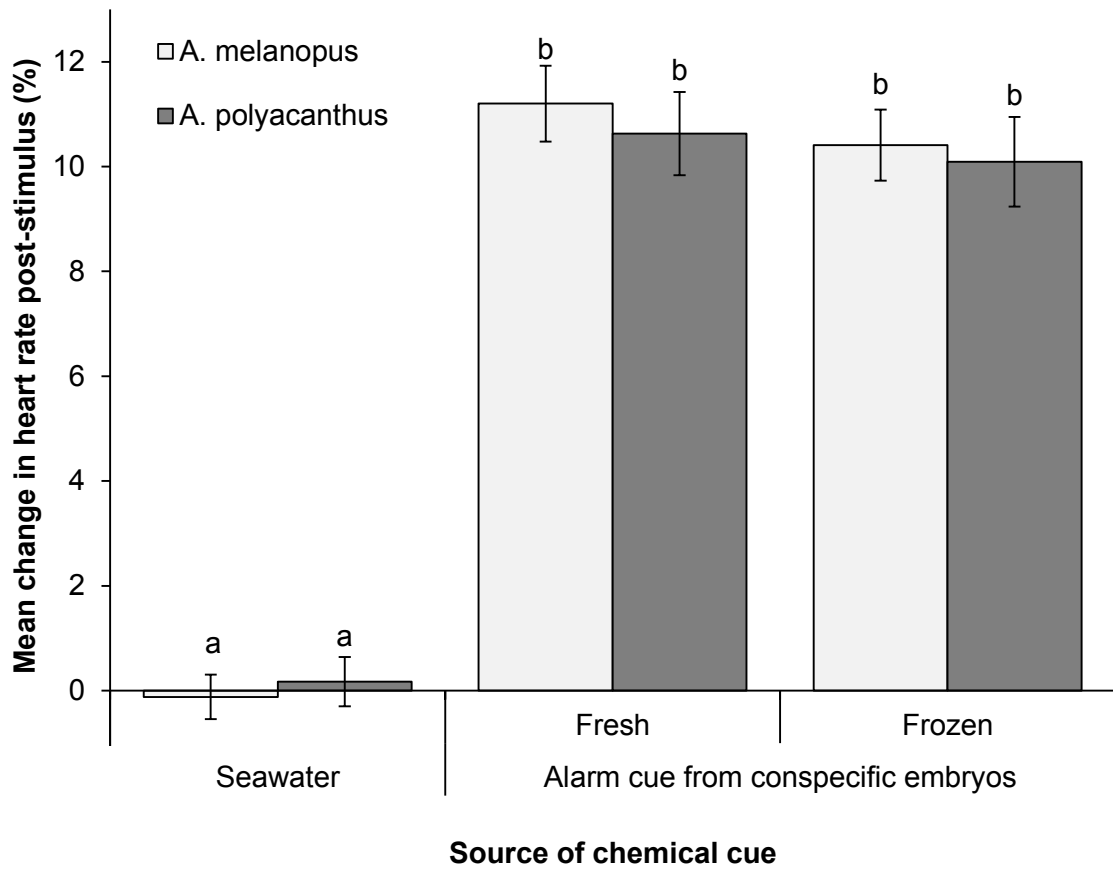


Figure A4.1: Comparison of the mean percentage change in heart rate (\pm SE) produced by the introduction of a chemical alarm cue produced from either fresh, or frozen and defrosted, conspecific embryos (for each bar $N = 45$). Letters denote Tukey's groupings of means.