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The Effects of High-risk Conditions on Coral Reef Fishes

Thesis submitted by

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General Abstract

Risk is one of the main drivers in shaping prey phenotypes around the world. Individuals that accurately assess risk have a better chance of mounting a suitable response to a threat cue and hence gain a selective advantage. Phenotypic plasticity in behaviour is advantageous especially for organisms that transition between stages of a complex life history, as it is hard to predict future risk. For coral reef fish, one such stage is known as settlement. At this stage, naïve juveniles are exposed to various levels of risk. Individuals that settle on high-risk habitats may develop a risk-adverse phenotype whereby novel cues are initially labelled as risky. There are many effects to having a risk-adverse phenotype that assist prey to increase their probability of survival. However, more studies need to be done to further understand the risk-adverse phenotype. This thesis aims to investigate: (1) How do high-risk conditions affect the fast-start escape response in juvenile coral reef fish; (2) How do high-risk conditions affect the morphology of juvenile coral reef fish. High-risk conditions were created by exposing individuals to conspecific chemical alarm cues three times a day for four days. Low-risk (control) conditions were created by exposing individuals to saltwater three times a day for four days.

Chapter 2 investigated the effects of high-risk conditions on the escape responses of a species of juvenile damselfish, the spiny chromis, *Acanthochromis polyacanthus* (Pomacentridae). Juvenile *A. polyacanthus* were caught using hand net on SCUBA and transported back to the laboratory. Individuals were then exposed to either a high-/low-risk treatment before being individually tested in a fast-start arena. Prior to the commencement of the test, individuals were exposed to either chemical alarm cues or saltwater to investigate the effect of acute stressors. All fish were filmed at 420 frames per second using a camera pointed at a mirror tilted at a 45° angle underneath the arena. This resulted in a silhouette of the moving fish. A principal component analysis (PCA) was used to analyse how variables measured (response latency, response duration, response distance, mean response speed, maximum response speed, maximum acceleration) differed between treatments. An analysis of variance (ANOVA) was also used to analyse how escape method and turning angle differed between treatments. Risk (background or acute) affected escape responses in two ways. Firstly, the method of escape used by individuals (i.e., C-start or backing away from the threat). Secondly, escape responses were enhanced by individuals exposed to high-risk (with/without acute risk) and low-risk with additional acute risk. Background risk and acute risk acted in a simple additive manner, as seen by the lack of interaction between the two factors. Results showed that escape responses are amplified as the level of perceived risk increases.

Chapter 3 investigated the effects of high-risk conditions on the morphology of a species of juvenile damselfish, the ambon damselfish, *Pomacentrus amboinesis* (Pomacentridae). Naïve fish leaving the pelagic phase to settle on reefs were caught by light traps and transported back to the laboratory where they were exposed to high-/low-risk conditions. Following background risk conditioning, individuals were taught to recognise a novel odour from a predator, the Brown dottyback, *Pseudochromis fuscus*, as risky or safe. Individuals were then housed in plastic containers for 28 days. During this period, individuals were exposed to the predator odour three times a day. Photographs were taken every seven days to document the change in body depth, standard length, ocellus area, ocellus diameter and eye diameter. Results found that individuals exposed to high-risk conditions grew larger ocelli after four days of exposure. At 28 days post risk history treatment, high-risk individuals not only had larger ocelli but significantly smaller eyes and a slightly larger body. Results suggest that exposure to predation risk at a critical time may place these fish on a certain growth trajectory based on experiences during their period of settlement.

This study demonstrates the importance of a risk-adverse phenotype for coral reef fishes settling onto high-risk habitats. As naïve juveniles are particularly vulnerable to predation at this stage in life, the risk-adverse phenotype increases the probability of survival. This is achieved by not only treating novel cues as a risk, but also altering morphology and escape responses to aid in the pre- and post-attack stages of an interaction with predators.

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Chapter 1: General Introduction

Predation risk

Predation risk is thought to be an important evolutionary driver underlying the astounding diversity in animal size, shape, colour and behaviour found in nature. Predator-prey interactions play a major role in shaping the life history characteristics of many species and as a result increase organismal complexity (Lima and Dill, 1990; Bengston, 2002). This strong selective pressure by predators shifts phenotypic diversity and alter the frequency of the underlying genes, thereby increasing the overall fitness of prey (Bengston, 2002; Planes and Romans, 2004). Predator-prey interactions can be conceptualised as a series of trade-offs (Lima and Dill 1990). Firstly, failure of prey to detect the predator increases the probability of capture during an event (Chivers et al., 2001). Secondly, to avoid predation, prey must develop an anti-predator response that typically comes at the expense of other fitness-enhancing activities (e.g., foraging and mating). Individuals that accurately assess risk have a better chance of mounting a suitable response to a threat cue (e.g., modifying its body shape or escape responses after the initiation of a predatory attack) and hence gain a selective advantage.

However, risk can be highly variable in space and time (Ferrari et al., 2009). Spatially, prey may live in habitats with an elevated population of predators, leading to greater levels of risk. For example, prey such as water buffalo, *Syncerus caffer*, kudu, *Tragelaphus strepsicerus*, and wilderbeast, *Connochaetes taurinus*, experienced higher predation risk at water sources compared to sites with dense vegetation (de Boer et al., 2010). Additionally, Pearman (1995) found pond size to increase the occurrence of a predatory beetle larvae which significantly affected the survival and metamorphosis mass of Western chorus frog tadpoles, *Psuedacris triserata*. Temporal variability in predation risk can occur on a range of scales, from seconds to months. For instance, the calcareous tubeworm, *Serpula vermiculates*, a filter feeder, rapidly retreats into its tube when startled by alarm stimuli (Dill and Fraser, 1997). For nocturnal prey, exposure to risk is correlated to the lunar cycle. For example, kangaroo rats, *Dipodomys merriami*, experience long periods of risk during full moon and they compensate for this by increasing their activity during dawn and dusk (Daly et al., 1992). Predation pressure can also vary seasonally with breeding seasons. Ryan (1985) found that when the male túngara frog, *Physalaemus pustulosus*, engages in mating calls (lasting for ~6 h) they are highly vulnerable to predation by bats.

Response to risk

Prey respond to the threat of predation at a number of biological levels (i.e., behavioural, physiological and morphological) through ecological and evolutionary time. Prior to a response, prey use a myriad of senses to aid in the detection of predators, such as vision (Pessoa et al., 2014), olfaction (Manassa and McCormick, 2012), hearing (Fournier et al., 2013) and mechanoreception (Hartman and Abrahams, 2000). Prey behavioural responses are limited by their physiology. Rather than physiologically coping with potential stressors, such as risk, prey will alter their behaviour. For example, mysids inhabiting deeper waters close to their lower limit of thermal tolerance to avoid high levels of predation (Boscarino et al., 2007). Physiological processes, such as stress responses that cause increased levels of catecholamines and glucocorticosteroids, drive morphology to assist in survival under the influence of stressors (Hawlena and Schmitz, 2010).

Prey use a variety of responses to protect themselves from predation when risk is high. Some examples include alarm calls (e.g., primates; Zuberbühler et al., 1999) and visual displays (e.g., push-up display of lizards: Leal and Rodriguez-Robles, 1996; or bobbing behaviours in fish: Smith and Smith, 1989), which signal to the predator that it has been detected. These signals can deter predators from attacking, as prey are less likely to be captured if they are aware of the predator's presence. In addition, prey can also alter their behaviour to reduce predator encounter rates and attack success (Lima and Dill, 1990). Zooplankton species reduce spontaneous activity levels in the presence of the predatory copepod, *Acanthocyclops vernalis* (Li and Li, 1979). Guppies, *Poecilia reticulata*, decrease their feeding rate in the presence of predatory cichlids (Fraser and Gilliam, 1987). Studies have shown that juvenile spiny chromis, *Acanthochromis polyacanthus*, modulate their escape responses (i.e., shorter latency to respond and faster swimming speeds) based on the perceived level of risk (Ramasamy et al., 2015). Finally, prey can alter their morphology to aid in escaping predators (Webb, 1977; Mikolajewski and Rolff, 2004). Hamill et al. (2009) found that hypotrich ciliates of the genus *Euplotes* increased their body width in the presence of a predator, a turbellarian of the genus *Stenostomum,* preventing this gape-limited flatworm from ingesting this prey. Similarly, the morphology of three-spined stickleback, *Gasterosteus aculeatus* is influenced by the type of predator to which they are exposed (Marchinko, 2009). Specifically, Machinko (2009) found insect predation resulted in shortened dorsal spines and pelvic girdle length. This lack of armour inhibits the predatory insect from constraining their prey during the attack allowing the prey to escape (Reimchen, 1980). Conversely, Reimchen (1994) found sticklebacks exposed to piscivourous fish and birds to develop long spines and more lateral plates to combat such gape-limited predators that have an upper limit on prey size due to their mouth width.

Neophobia: the risk-adverse phenotype

As risk can vary on spatial and temporal scales, prey have to be able to alter their phenotype to best respond to their particular habitat. Recent research has focused on the development of an alternative, risk-averse phenotype in prey known as neophobia. Neophobia is the result of an individual being exposed to high levels of risk (i.e., habitats with a large predator population). Neophobic prey exhibit an aversion to novel stimuli, making them less willing to execute risky behaviours in novel situations. Early studies on neophobia focussed on laboratory rats that only ate food from a familiar container instead of a novel container (Mitchell et al., 1973). Current research has focused on the effects of high levels of background risk on the development of neophobia in aquatic vertebrates (i.e., amphibians and fishes). Neophobia was induced by simulating a high-risk environment using chemical alarm cues from damaged conspecifics over multiple consecutive days (Brown et al., 2013; Chivers et al., 2014; Ferrari et al., 2015a). Chemical alarm cues are chemical compounds released from the prey's epidermis upon damage, representing a reliable indicator of the presence of a direct threat in the immediate area (Ferrari et al., 2015a). Once aware of the high-risk nature of their environment, animals treat any unknown cue as a potential risk. Brown et al. (2013) catalogued the first case of risk-induced neophobia in convict cichlids, *Amatitlania nigrofasciata*, and wood frog tadpoles, *Rana sylvatica*. Through the reliance on neophobic responses, naïve prey are more likely to survive the first encounter by avoiding novel predators (Ferrari et al., 2015a). This can be especially important during transitional phases, where animals move from one environment to another (e.g., metamorphosis from tadpoles to frogs or settlement stages in coral reef fish).

The benefits of neophobia

Neophobia can create an initial survival advantage in risky habitats, as animals that stay close to shelter are usually less vulnerable to predators (e.g., McCormick, 2009). This cautiousness is only beneficial for a short period of time, as prolonging neophobia will lead to loss of foraging opportunities. During this risk-averse state, animals can gain knowledge on the identity of predators in three primary ways. First, animals can have a direct interaction with a predator and survive the strike. Given that the strike efficiency of piscivores is low (e.g., <14% in common coral reef predators, Feeney et al. 2012), this can be a risky but effective method of learning. Second, fish can learn about predators when conspecifics are attacked, by concurrently detecting chemical alarm cues and olfactory cues of the predator (Ferrari et al., 2010). Lastly, individuals can use public information, either by directly witnessing a predation event or by mimicking the evasive behaviours of conspecifics through social learning (Manassa and McCormick, 2012). Aquatic vertebrates (i.e., fish and amphibians) possess a sophisticated and dynamic way of allocating risk to particular cues. The more frequently a cue is paired with a reliable risk indicator (e.g., an alarm cue, or antipredator response of a neighbour), the greater the risk that the cue is assigned (McCormick and Manassa, 2008). On the other hand, the importance of other cues can be reduced if they occur without any form of risk reinforcement (e.g., no visual cue of a predator attack or olfactory alarm cue), a process known as latent inhibition (Mitchell et al., 2011). Thus, fish have rapid and effective methods of learning risk and a risk-averse, neophobic behavioural state can greatly reduce the vulnerability of naïve prey.

By becoming neophobic, naïve prey can also reduce their initial cost of learning (i.e., learning predator identity while risking death). Chivers et al. (2014) found that juvenile damselfish, *Pomacentrus chrysurus,* that had been exposed to alarm cues and developed a neophobia could not use latent inhibition to downregulate their reaction towards cues. This suppression of latent inhibition prevents incorrect labelling novel cues, which could potentially be risky, as safe. Interestingly, neophobia can also be transferred between conspecifics. Crane et al. (2015) used fathead minnows, *Pimephales promelas*, to demonstrate that fish can observe the behaviour of conspecifics from high-risk conditions and subsequently display some neophobic responses to novel odours.

Neophobia can also influence fish physiology. Ferrari et al. (2015a) found that fish accustomed to high-risk conditions recover metabolically from stress more quickly than their low-risk counterparts. This ability to reach resting metabolic rates faster may minimise the cost of reacting to stimuli and allow prey to resume other fitness promoting behaviours (e.g., feeding and defence) sooner. Neophobia has also been found to modify the behaviour (i.e., escape responses) of *P. chrysurus*. Fish raised within high-risk conditions were ~ 60 % more lateralised (i.e., turned in one direction more often than the other) and had shorter latencies (i.e., time taken to respond to stimulus) than low-risk counterparts. This is beneficial, as individuals would increase their probability of survival as they exhibit more effective decision making during the escape response. These changes may occur as the perceived level of risk in individuals is elevated in high-risk conditions (Ramasamy et al., 2015). This amplification of the escape responses increases the chance of surviving predatory attacks in high-risk fish (Ferrari et al., 2015a). Neophobia also improves survival, with both laboratory and field studies finding higher survival in neophobic fishes than non-neophobic fishes (Ferrari et al., 2015a; Ferrari et al., 2015b; Ferrari et al., 2015c).

Thesis outline and aims

Though previous research illustrates the importance of an individual's history of risk, there are still many unanswered questions. Risk can change between habitats and across ontogenetic stages. Therefore, animals are not uniformly susceptible to perturbations throughout their lifetime, and will therefore likely exhibit greater sensitivity to specific types of risk at certain time points. For marine organisms that have complex life histories with a dispersive larval phase, settlement to the juvenile habitat represents a critical transition marked by high and variable mortality (Leis and McCormick, 2002). During this settlement period (McCormick and Makey, 1997), an individual's physical and behavioural phenotype determines its chance of survival (Sogard, 1997; Holmes and McCormick, 2009). Therefore, it is critical to understand how individuals that settle into high-risk habitats modulate predatory risk. Recent research has uncovered the physiological changes of individuals under high-risk conditions. Ferrari and colleagues (2015a) investigated the effects of high-risk conditions on the initial stages on the escape response (i.e., latency). An escape response, otherwise known as a fast-start, is short, high-energy swimming burst that is powered anaerobically (Domenici and Blake, 1997). Escape responses consist of three discrete stages: the formation of the c-bend (stage 1), the propulsive stroke (stage 2), and continuous swimming or coasting (stage 3). It is as yet unknown how other aspects of the escape response during stage 3 (i.e., response distance, response speed, and turning rates) are affected. Additionally, little is known on the effects of high-risk conditions post-settlement on the morphology of juvenile coral reef fishes. Literature has several examples of prey altering their morphology as a result of prolonged exposure to predators. For example, the crucian carp, *Carassius carassius*, was found to develop a deeper body in response to chemical cues from piscivores (Nilsson et al., 1995). However, as documented in previous literature, short-term exposure to elevated risk levels can lead to the development of the risk-adverse phenotype. Therefore, the aim of this thesis is to investigate two knowledge gaps: (1) How do high-risk conditions affect the fast-start escape response in juvenile coral reef fish; (2) How do high-risk conditions affect the morphology of juvenile coral reef fish.

Chapter 2: Juvenile coral reef fish alters escape responses when exposed to changes in background and acute risk levels

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2.1 Summary

The response of prey to predation threats is often plastic and can vary with the individual's perceived level of threat. To determine if prey escape responses can be modulated by background levels of risk or short-term acute risk conditions, juvenile damselfish were maintained under high- or low-risk background for 4 days and then exposed them to an acute risk indicator (i.e., alarm cues [high-risk] or a saltwater control [low-risk]) minutes prior to being startled with a mechanical disturbance. Fish undertook one of two responses: a C-start escape response or they back away from the threat. While exposure to either high background or acute high-risk increased the proportion of C-starters, the frequency of C-starters decreased when high background and acute risk types were combined. Regardless of response type, acute high-risk always improved escape performance of the fish. High-risk conditions, both background and acute, also improved performance, but this enhancing effect was only occurring for C-starters. Non C-starters reacted similarly in both background risk conditions. Background risk and acute risk acted in a simple additive manner, as seen by the lack of interaction between the two factors. Results showed that escape responses are amplified as the level of perceived risk increases.

2.2 Introduction

Predation risk plays a major role in shaping prey populations. Predators can impact prey through direct interactions resulting in mortality. However, they can also have an indirect effect influencing life-history characteristics (Bernard, 2004; Preisser et al., 2005; Palacios et al., 2016). Moreover, an organism's history of risk can shape its morphology (Brönmark and Petterson, 1994; Chivers et al., 2008) and behavioural phenotype (i.e., response to predators) with direct repercussions on survival (Lönnstedt et al., 2013). For example, a history of high risk during early ontogeny can result in overly cautious behaviour when presented with novel stimuli, a condition known as neophobia (Brown et al., 2013). For marine organisms that have a bipartite life cycle, the transition to juvenile habitat is particularly critical and is marked by high mortality (Almany and Webster, 2006). During this period the physical and behavioural phenotype of individuals strongly influences survival (Sogard, 1997; McCormick and Meekan, 2010; McCormick and Holmes, 2010). Being risk-adverse or neophobic is highly beneficial when entering new habitats (Ferrari et al., 2015) particularly when confronted with a myriad of new predators.

Predator-prey interactions follow a well-described sequence of events, from detection to capture or escape. Within this sequence, there are steps where both the predator and prey can optimise their success (Lima and Dill, 1990; Domenici and Blake, 1997). Our study focuses on the crucial step when prey must escape from a predator after an attack has been initiated. Escape responses, referred to as fast-starts, are short, anaerobically powered, swimming bursts elicited by the activation of a large pair of reticulospinal neurons called the Mauthner cells (Moyes et al., 1993; Domenici and Blake, 1997).

To undertake a successful escape, prey will use all information available to them. This information can be visual (i.e., sight of a predator), chemical (i.e., predator odour or chemical alarm cues) or both. Behavioural history (i.e., prior experiences that affect future behaviour) has also been shown to affect the mechanics (i.e., kinematics) of the fast-start response (e.g., Langerhans et al., 2004; Ramasamy et al., 2015) and suggests that rather than being hardwired, components of the fast start response are under cognitive behavioural control (Ramasamy et al., 2015) and are context dependent (Domenici, 2010; Chivers et al., 2016; McCormick and Allan, 2016). Given the strong influence of predation history on behaviour and the development of risk-adverse phenotypes (Ferrari et al., 2015a) our present study examines the role that background risk and acute risk have on escape responses. To do this, we used the coral reef fish, spiny chromis, *Acanthochromis polyacanthus*, to ask: (1) Does background risk history influence an individual's escape performance; (2) Does the addition of information on current (i.e., acute) risk affect the escape responses of individuals exposed to different risk histories? To test this, juvenile fish were captured, and given two background risk treatments using damaged released cues from conspecifics (i.e., alarm cues), known to elicit an antipredator response in this species (Manassa and McCormick, 2012). Minutes prior to being startled, these fish were also exposed a low- or high-risk stimulus.

2.3 Methods

Study Species

Five schools of juvenile *A. polyacanthus* (Pomacentridae) (18.37mm ± 0.21mm), a reef-associated brooding planktivore commonly found on the Great Barrier Reef (GBR), Australia, were captured using hand nets and clove oil while on SCUBA near the reefs surrounding the Lizard Island Research Station (14°40'S, 145°28'E), northern GBR in March 2015. The fish were transported to the laboratory and held in 32-L flow-through tanks (43 x 32 x 31 cm), where they were fed *Artemia sp.* three times per day for four days.

Conditioning Regime

The goal of this experiment was to test the effect of background and acute risk on the escape response of a coral reef fish. We crossed background risk (low vs high) with an acute risk treatment (low vs high) in a 2 x 2 design. To establish the high-risk background, we followed the procedures of Chivers et al. (2014). Fish (n = 72) were equally divided into a series of 12, 3 L tanks. Fish in half of the tanks were exposed to elevated risk for four days while the remainder were exposed to a low-risk control. High-risk conditions were created by introducing a solution of alarm cues into the conditioning tanks three times per day for four days. The alarm cue solution was prepared minutes prior to being used, by making six vertical cuts on each side of four, freshly euthanized (using cold shock, in accordance with James Cook University animal ethics guidelines, permit: A2005), donor conspecific fish and then rinsing the fish in 60 mL of saltwater. We injected 10 mL of this alarm cue solution into the conditioning tanks, which gave a concentration of 2 cuts/L once injected. This concentration has been shown to elicit strong antipredator responses in coral reef fishes (Chivers et al., 2014; Ferrari et al., 2015a). The timing of the three injections occurred randomly between 0800 and 1800 h, with a minimum of 1.5 h between consecutive injections. Low-risk conditions were obtained by injecting 10 mL of saltwater on the same time schedule as the high-risk treatment.

Fast-start Assay

After fish had been in one of the two risk treatments for four days, we conducted a fast-start assay to test if the response of fish was affected by background risk or the presence of current acute risk (i.e., alarm cues). Alarm cues were produced fresh (2 cuts/L). Fish were given a 3-min acclimation within the test arena prior to the introduction of 20 mL of either high- (i.e., alarm cue) or low-risk (i.e., saltwater). The fish were exposed to the cue for two minutes before an escape response was elicited. We followed the methods described in other fast-start studies (Allan et al., 2013; Ramasamy et al. 2015) in which a tapered metal weight was released from above the water surface. The metal weight was controlled by a piece of fishing line that was long enough to allow the tapered tip to lightly touch the surface of the water but not hit the bottom of the tank. In order to remove the possibility of fish responding to the visual cue of the approaching stimulus, the weight was released through a white PVC tube (4 cm diameter x 55 cm length) suspended above the experimental arena, with the bottom edge sitting 1 cm above the water level.

To standardise the distance between the test subject and the stimulus, fish were only startled when they moved to the middle portion of the tank. This also allowed the individual to move in any direction. Escape responses were recorded at 480 frames per second as a silhouette from below obtained through pointing the camera (Casio EX-ZR1000) at a mirror angled at 45˚. The water in the experimental arena was changed after each trial to avoid a potential build-up of alarm cues across trials. The following kinematic variables were measured:

1) Response latency (s) was measured as the time interval between the stimulus touching the water surface and the first detectable movement of the fish.
2) Response duration (s) was measured as the elapsed time from the start to the end of the escape response (i.e., when the fish comes to a halt).
3) Response distance (m) is a measure of the total distance covered by the fish from the onset of the escape response to the end (i.e., when the fish comes to a halt).
4) Mean response speed (m s^{-1}) was measured as the distance covered within a fixed time (first 29 ms after the initial response) which corresponds to the average duration of the

first two tail flips (the first two axial bends, i.e., stages 1 and 2 based on Domenici and Blake (1997)). This period is considered crucial for avoiding predator ambush attacks (Domenici and Blake, 1997).

5) Maximum response speed (m s^{-1}) was measured as the maximum speed reached at any point during the escape response.
6) Maximum acceleration (m s^{-2}) was measured as the maximum acceleration within a fixed time (first 29 ms after initial response).
7) Turning angle (°) was measured as the total angle created during stage 1 of the response between the head to the middle of the fish.

Statistical Analysis

Data was split into 2 groups: C-starters (i.e., commencement of the escape response resulting in the individual forming a C-shape) and non C-starters (i.e., fish that swam backwards away from the stimulus). Given the variation in the occurrence of C-start responses, we first conducted a logistic regression testing the effect of background risk (low vs. high) and acute risk (low vs high) on the occurrence of C-start and non C-start responses (binomial data).

Given the high level of correlation among the kinematic variables ($r > 0.38$), latency, mean escape speed, maximum escape speed, maximum acceleration and response duration and response distance were reduced to two synthetic variables explaining 83% of total variance using a correlation matrix-based PCA. The first eigenvector, which explained 66% of the variance, loaded heavily and positively on the speed and acceleration variables and negatively on latency (Table 2.1). We coined this synthetic variable the “velocity component” of the escape response. The second eigenvector explained 17% of the variance and loaded primarily and positively on response duration and distance, and we referred to this variable as the “effort component” of the response. These two orthogonal variables were used to test the effect of background and acute risk on the escape performance of the fish. In addition, we also included response type (C-start vs non C-start) as a factor in the analysis to tease apart variation due to response type (C-starts are typically faster) and risk treatments.

Finally, the turning angle variable, available only for fish performing C-starts, was analysed using a 2-way ANOVA to examine the effect of background and acute risk on the response. The data were square-root transformed to improve normality.

Table 2.1: Principal component analysis loadings for component 1 (66%) and component 2 (17%).

	Component 1	**Component 2**
Latency	-0.701	0.114
Response duration	-0.650	0.688
Response distance	0.637	0.721
Mean response speed	0.971	0.002
Maximum acceleration	0.875	0.016
Maximum response speed	0.966	0.053

2.4 Results

Escape Type

The dichotomy between escape types did not appear in the overall analysis as the increased duration and increased distance both loaded positively on PC2, but were opposite on PC1: distance was positively correlated with PC1 scores, while duration was negatively correlated with PC1. There was a significant interaction between background and acute risk on the frequency of fish displaying C-start responses when startled (Wald χ^2_1=11.1, P = 0.001, Fig. 2.1). Namely, when low-risk fish were exposed to high acute risk when startled, they were more likely to C-start than those exposed to water only (acute risk: Wald χ^2_1=4.9, P = 0.028). However, when high-risk fish were startled, those exposed to high acute risk were less likely to C-start (acute risk: Wald χ^2_1=6.2, P = 0.012).

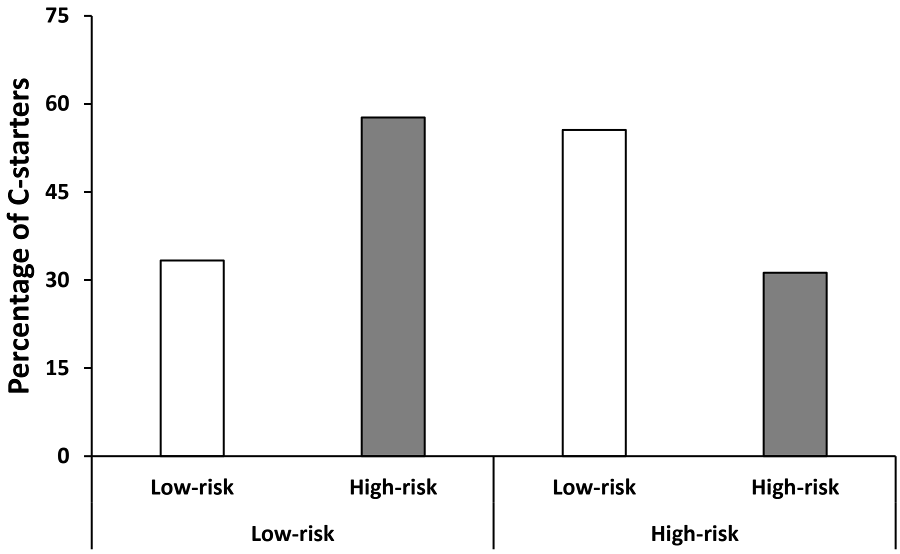

Figure 2.1: Percentage of fish displaying C-start responses within each treatment group. Fish were maintained under low-risk or high-risk conditions for 4 days (background risk), and were then exposed to an acute stressor (saltwater – empty bars, or alarm cues – grey bars) minutes prior to being startled (n = 15-25/treatment).

Velocity Component of the Escape Response (PC1)

Unsurprisingly, the velocity component of the C-starters' escape response was significantly better than those of non C-starter (3-way ANOVA, response type: $F_{1, 72} = 168$, $P < 0.001$, Fig. 2.2). Also, fish performed better in the presence of alarm cues (acute risk: $F_{1, 72} = 4.5$, $P = 0.038$), regardless of the type of response they displayed (response type x acute risk: $F_{1, 72} = 2.2$, $P = 0.14$), in both background risk levels (response type x acute risk x background risk: $F_{1, 72} = 0.5$, $P = 0.47$). The effect of background risk on escape performance depended on the type of response used (background risk x response type: $F_{1, 72} = 8.0$, $P = 0.006$), but not the acute risk level (background risk x acute risk: $F_{1, 72} = 0.2$, $P = 0.65$). High-risk background risk exposure lead C-starters to perform better than those kept under low background risk (post-hoc: $F_{1, 34} = 7.7$, $P = 0.009$) but this enhancing effect did not occur for non C-starters (post-hoc: $F_{1, 38} = 0.1$, $P = 0.8$).

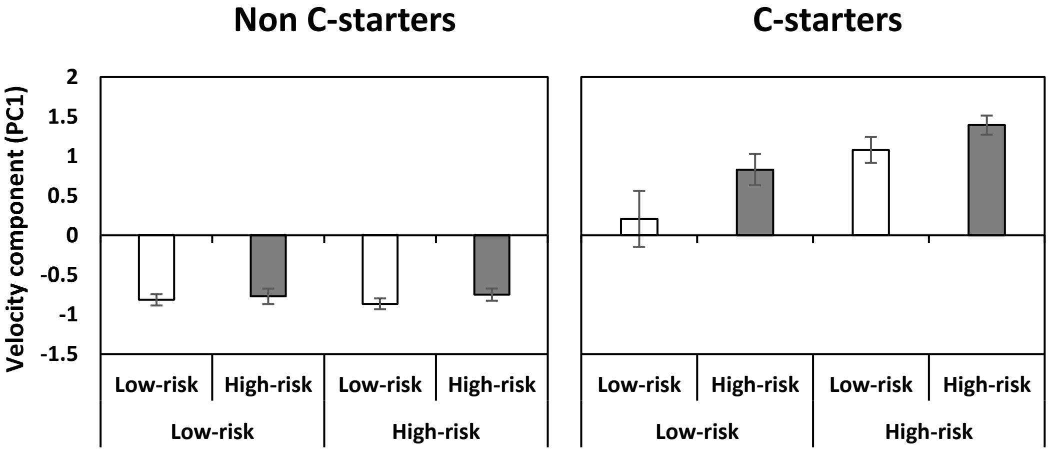

Figure 2.2: Mean ± SE scores from the first eigenvector (PC1 – explained 66% of total variance) loading on the speed and acceleration aspect of the escape responses. Fish were maintained under low-risk or high-risk conditions for 4 days (background risk), and were then exposed to an acute stressor (low-risk – empty bar, or high-risk – grey bars) minutes prior to being startled.

Effort Component of the Escape Response (PC2)

Overall the effort component of the escape was improved in the presence of alarm cues (3-way ANOVA, acute risk: $F_{1,\,72} = 6.3$, $P = 0.015$, Fig. 2.3), but this effect was not dependent on background risk (acute risk x background risk: $F_{1,\,72} = 1.4$, $P = 0.24$) or the type of response performed (acute risk x response type: $F_{1,\,72} = 0.1$, $P = 0.7$), or any interaction among those three factors (acute risk x background risk x response type: $F_{1,\,72} = 0.3$, $P = 0.9$). The enhancing effects of high-risk background risk only occurred for C-starters (background risk x response type: $F_{1,\,72} = 8.9$, $P = 0.004$), which demonstrated improved performance when compared to their low-risk counterpart (post-hoc: $F_{1,\,34} = 8.7$, $P = 0.006$), while the performance of non C-starters was not affected by background risk levels (post-hoc: $F_{1,\,38} = 2.5$, $P = 0.12$). Individual analysis of the response distance variable show that fish from high background risk conditions showed a significantly greater response than fish from low background risk conditions ($F_{1,\,29} = 5.82$, $P = 0.02$; supplementary figure 1).

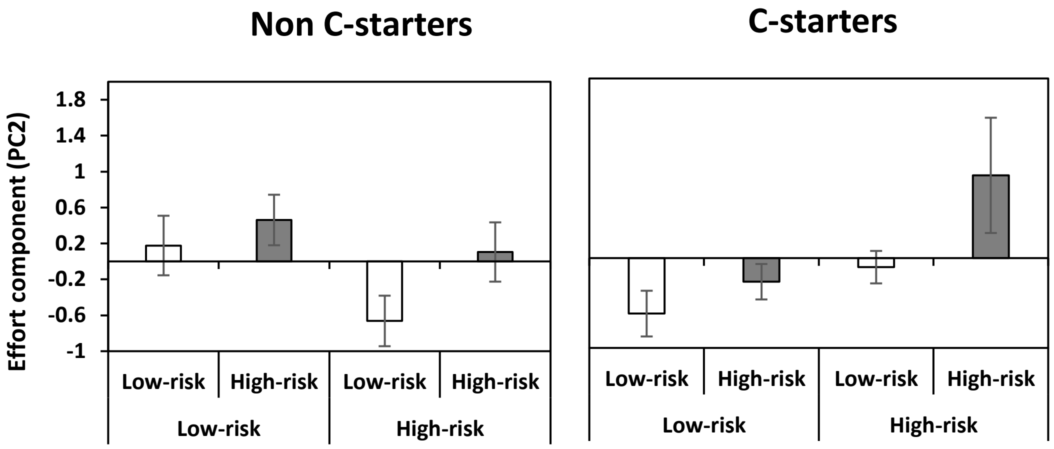

Figure 2.3: Mean ± SE scores from the second eigenvector (PC2 – explained 17% of total variance) loading on the duration and distance achieved during the escape. Fish were maintained under low-risk or high-risk conditions for 4 days (background risk), and were then exposed to an acute stressor (low-risk – empty bars, or high-risk – grey bars) minutes prior to being startled.

Turning Angle

The 2-way ANOVA revealed no statistical difference in the turning angles of the fish performing C-starts (background risk: $F_{1,\,34} = 1.9$, $P = 0.18$, acute risk: $F_{1,\,34} = 1.9$, $P = 0.18$, background risk x acute risk: $F_{1,\,34} = 4.0$, $P = 0.0053$, n = 4-15/treatment, Fig. 2.4).

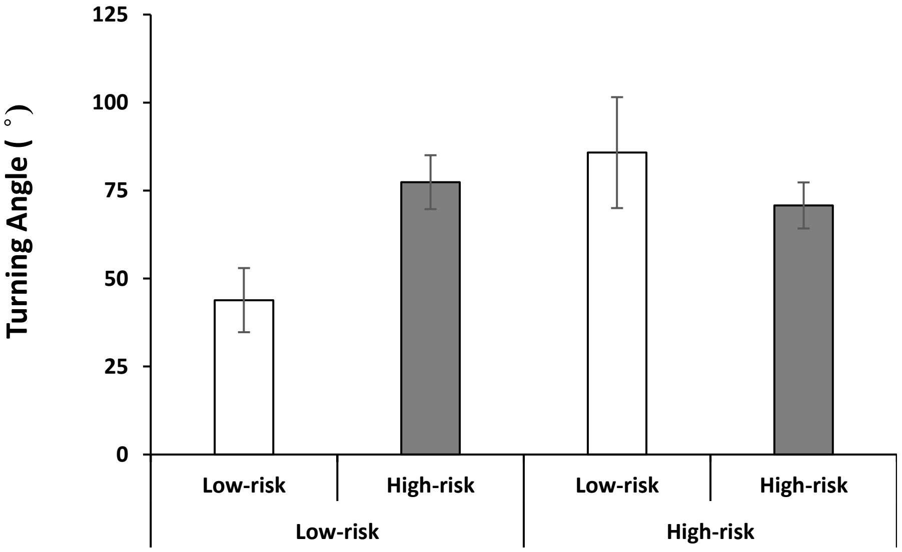

Figure 2.4: Mean ± SE turning angle of fish performing C-starts. Fish were maintained under low-risk or high-risk conditions for 4 days (background risk), and were then exposed to an acute stressor (saltwater – empty markers, or alarm cues – grey markers) minutes prior to being startled.

2.5 Discussion

Our results revealed a surprising sophistication in the way risk perception modulates escape responses. We found an intriguing pattern of responses when it came to fish displaying C-starts, the most effective way to avoid a motivated predator. Fish exposed to high-risk, whether through background risk treatment or acute exposure, increased the frequency with which they displayed C-starts, when compared to low-risk controls. This result seems logical as C-starts, which are faster in nature than other escape responses, may improve survival odds (O'Steen et al., 2002). However, when presented with both stressors, the proportion of fish displaying C-starts was lower, possibly due to the acclimation to the high-risk environment and a change in the way high-risk fish optimise their escape response.

Fish exposed to both high-background and acute risk are likely expecting an imminent attack. Due to the high costs of disrupting normal daily activities, fish from high-risk environments may benefit from setting the threshold for a fast-start higher than in a low-risk environment. Hence fish under high-risk background may require more reliable indicators of attacks before C-starting than fish used to a low-risk environment. When we investigated the components of their escape responses, we first found that, as expected, C-starters displayed responses that were faster, in terms of response speed when compared to non C-starters (supplementary figure 1). The effort component, which encompassed response duration and distance, was affected by both background risk and acute risk conditions, in an additive manner. When looking at each component of this response (supplementary figure 1), it is clear that the highest risk group (i.e., high-background + acute risk) displayed C-start responses with the shortest latencies that were among the fastest and longest responses among C-starters, resulting in an escape distance being significantly longer than other groups. The enhanced response would increase the chances of survival from predatory attacks (Ramasamy et al., 2015). This supports Lima and Dill's (1990) risk allocation hypothesis. They explained that animals make decisions based on its reflection of the trade-offs for a given activity. Thus, when exposed to alarm cues prior to being startled, the high-risk individuals that backed away instead of performing a C-start may perceive the risk of the alarm cue to be insufficient to employ the high-energy swimming burst. A previous study on Western mosquitofish, *Gambusia affinis*, coexisting with predators showed individuals performed C-starts more often in environments with predators than their *G. affinis* from predator-free populations (Langerhans et al., 2004). Another factor to take into account might be that fish did not perceive the threat of the stimulus weight as strong enough. Therefore, the action potential generated by the Mauthner cell was weak resulting in a reduced escape response.

Risk has been shown to affect the physiology of fishes, in particular their levels of stress hormones such as cortisol, and the way they partition energy. A recent review of the influence of predator pressure on the hypothalamus-pituitary-adrenal/hypothalamus-pituitary-interrenal axis (HPA/HPI) indicates that, while acute exposure to predation risk elevates cortisol in most published instances (72 of 99 studies), this is only the case for 50% (36 of 68 studies) of the cases where vertebrates are exposed to long-term predation threat (Harris and Carr, 2016). Chronic activation of the sensory pathways that inform the HPA/HPI axis about predators may result in classical habituation of neuronal pathways and consequently behavioural responses (Grissom and Bhatnagar, 2009). This may have occurred for some individuals in response to our high-risk treatment; a high level of individuality in adrenocortical responsiveness and the capacity to habituate to chronic stress was found in rats (Natelson et al., 1988) and this may also be the case for fishes. Juvenile coral reef fishes have extremely high metabolic rates (Nilsson et al., 2007) and there is likely to be a strong selective pressure to be adaptable to predation threat as reductions in foraging on time scales of hours may have major repercussions for growth and survival due to their relatively low energy reserves. Thus, a risk-driven change in energy allocation may affect the algorithm used to determine when to maximise their fast-start response, in keeping with the “Economic Hypothesis” described by Ydenberg and Dill (1986).

For the fish that did not display C-start responses, we observed a different overall strategy of swimming backwards (supplementary figure 1). For non C-starters, their escape responses were affected by acute risk conditions, but not background risk. Typically, fish exposed to high acute risk (i.e., alarm cues) responded for longer and escaped further than those exposed to low acute risk (i.e., saltwater). This may be because the non C-starters represent those fish that have habituated to the high-risk background environment and have effectively reset their judgement of baseline risk. Mirza et al. (2006) found juvenile rainbow trout, *Oncorhynchus mykiss*, that were exposed to risky conditions regularly responded with significantly reduced antipredator responses. By backing away from their original position, non-C-starters may be attempting to maintain visual contact with the source of the disturbance. Additionally, the lack of a response could be attributed to physiological stress. In treatments involving high-risk fish paired with alarm cue, these individuals may have increased levels of cortisol such that it becomes detrimental to their performance (Maule, 1988; Schreck, 2000). Deepening our understanding of the physiological mechanisms responsible for the variations in escape strategy performances may provide some insights into the impact of environmental factors (i.e., food, risk, etc.) on such variation.

Overall, our findings support the notion that escape responses have an inherent level of plasticity that could be driven by abiotic and biotic environmental factors, such as predation. A recent study demonstrated that spiny chromis, *A. polyacanthus*, adjust their level of responsiveness in their fast-start to the perceived level of current risk from the sight or smell of a known predator (Ramasamy et al., 2015). Another damselfish, *Pomacentrus chrysurus* has also been shown to optimise its fast-start kinematics based on information concerning the identity of specific predators (McCormick and Allan, 2016). Our results provide further evidence that escape responses are highly plastic, context dependent and species-specific (Webb, 1986; Domenici and Batty, 1997; Domenici, 2010; Ramasamy et al., 2015; McCormick and Allan, 2016). While stress (i.e., cortisol level) could be a proximate mechanism for the variation seen, the relationship between stress and performance is unlikely to be linear (Schreck, 2000). Fish from high-risk treatments may have had greater levels of cortisol priming their flight and fight response system (Henry, 1993). As previously mentioned, those in the highest risk treatment may have increased levels of cortisol reaching a level that is detrimental to their performance (Maule, 1988; Schreck, 2000). However, the conditions we created would not exceed the predation situation in the wild, where such juvenile fish are exposed to a myriad of predators much more often than three times a day. It is possible that fish that failed to match threats with an appropriate response (i.e., an optimised C-start when there is extreme risk) could be easily preyed upon in nature. The notion that a high-risk background results in lower responses is already a well-established theory in the form of the Risk Allocation Hypothesis (Lima and Dill, 1990, Ferrari et al., 2009). This model predicts that prey exposed to increased risk, both in intensity or frequency, cannot afford to increase their response intensity linearly, as other competing demands, like foraging, would take precedence over hiding. Instead, prey allocate their maximum antipredator effort during periods of highest perceived risk, and maximise foraging efforts during periods of highest perceived safety. This ‘perceived safety’ is what can be confusing, as one individual’s perception of safety in a particular habitat at a particular time, might be another individual’s highest risk period, making the two responses appearing contradictory. The apparent decrease in performance from the highest risk groups might be couched in the light of the Risk Allocation, as an arena devoid of visual predation risk

– the sensory input with the highest level of reliability – might be considered a ‘safe’ place for predation-primed fish.

Results from this study have highlighted the effects of varying levels of risk on escape responses, with background level of risk affecting some responses types but not others, while acute risk appeared to universally increase fish performance in response to a weight stimulus drop. However, there is little information pertaining to how varying risk histories (e.g., 2d or 8d of high-risk instead of 4d) and ontogenetic stages (i.e., adults) affect escape responses. Escape responses may be further influenced by these variables as they influence an individual’s perceived level of risk. In a spatially and temporally variable environment that is a coral reef, it would be interesting to uncover how changes to one’s risk regime influences escape responses.

Chapter 3: Critical risk window in early life affects development of predator induced morphology of a coral reef fish

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3.1 Summary

Predation is a strong evolutionary force that alters the behaviour, physiology and morphology of individuals with population, community and ecosystem level effects. For many organisms the threat of predation alone is sufficient to alter growth and developmental trajectories, resulting in morphologies that reduce the probability of death in a high risk environment. For coral reef damselfish, one such trait is to grow deeper bodies to increase escape responses through higher acceleration and manoeuvrability. Another is to grow a false eyespot, known as the ocellus. Used as a diversion technique, it draws attacks of the predator towards non-vital regions of the body. In habitats with high levels of risk, organisms may develop an alternative phenotype, known as neophobia. Neophobic individuals are known to treat novel stimuli as a risk to avoid possible unknown threats. Many studies have focused on the behavioural aspects of individuals raised in high-risk habitats, but how early risk influences morphological development is unknown. In this study, we collected pre-settlement juvenile damselfish, *Pomacentrus amboinensis*, and exposed them to either injured conspecific cues (i.e. high-risk condition) or saltwater (i.e. low-risk condition) for 4 days before being training them to recognise the odour of a predator, *Pseudochromis fuscus*. The fish were then grown in individual containers for 28 days post-treatment with half being exposed daily to predator odour (threat cues) in a 2 x 2 design (risk history x current risk). Results indicate that fish with a high-risk history grew larger ocelli and smaller eyes compared to their counterparts raised under low-risk conditions, regardless of their current level of threat. Additionally, high-risk individuals grew slightly larger and longer than low-risk fish. Our results suggest that exposure to predation risk at a critical time may place these fish on a certain growth trajectory based on experiences during their period of settlement.

3.2 Introduction

Throughout evolutionary time predator-prey interactions have been part of a process that shape not only the behaviour, but the morphology of organisms (Lima, 1998). As predation pressure increases, prey are exposed to higher levels of risk whilst performing fitness-related behaviours, such as foraging and mating. The resulting effects of this predation pressure are changes allowing individuals to either recognise or evade predators. As this pressure is highly variable within space and time (Sih et al., 2000; Ferrari et al., 2009) it may be beneficial for prey to exhibit phenotypic plasticity, such as behavioural or morphological changes, to be able to survive the varying threats. An example of this plasticity can be seen in the alteration of foraging location and time to avoid encounters with a predator (Lima and Dill, 1990). Certain animals, such as amphibians, have been known to hatch and/or metamorphose earlier or later depending on the number of egg predators or larval predators they can detect in their habitat (Sih and Moore, 1993). These examples of phenotypic plasticity allow prey to maximise the trade-offs between anti-predator responses and fitness related activities, such as foraging and mating (Ferrari et al., 2009).

Organisms with complex life cycles, such as amphibians and fishes, undergo ontogenetic shifts in habitats as they transition from larval to juvenile stages, or from nursery to adult environments. This exposes these organisms to a diverse and changing array of predators. The general lack of parental care and intergenerational information also means that they have developed sophisticated ways of identifying predators and updating information on current risk. This information allows them to adjust their physiology, behaviour and morphology to the form best suited to survival.

In the case of prey in coral reef ecosystems, individuals may face a wide variety of predators. The threat of predation is especially high during the settlement stage where on average 60 % of juveniles are consumed within the first two days (Almany and Webster, 2006). As juveniles settle onto the reef, they have to quickly learn about their potential predators and alter their phenotypes based on their experiences. Prey use different sensory systems such as visual (Lönnstedt et al., 2012), auditory (Kelley and Magnurran, 2003), and olfactory (Hartman and Abrahams, 2000) to detect potential predators. After detection, learning is carried out through one of two methods. Firstly, through using first hand visual and/or olfactory stimuli, using the sight and/or scent of a predator coupled with chemical alarm cues (i.e. chemical compounds released from the epidermis of a fish upon damage), known as associative learning (Ferrari et al., 2010). The second form of learning, called social learning, occurs when inexperienced members of a group copy and remember the anti-predator behaviour of experienced counterparts (Manassa and McCormick, 2012). Studies have shown that prey are able to use these experiences to alter anti-predator behaviours. For instance, many studies have shown individuals that are taught to identify predators, exhibit not only a reduced feeding rate but a decrease in swimming activity when exposed to learned risky cues (Lönnstedt et al., 2013). Additionally, these experienced individuals have shown increased escape responses (i.e. shorter latencies and higher swimming speeds) as threat levels increase (Ramasamy et al., 2015, McCormick and Allan, 2016).

Recently, research has focussed on the effects of a high background level of risk on aquatic vertebrates. Studies have found an alternative risk-adverse phenotype, known as neophobia, in both amphibians and fish when individuals are exposed to alarm cues from damaged conspecifics for multiple days (Brown et al., 2013). Neophobia is a behaviour whereby individuals label novel stimuli as risky (Chivers et al., 2014; Ferrari et al. 2015a; Crane et al., 2015). It is believed that neophobic individuals initially fear novel stimuli before confirming real threats in their environment to survive the critical phases of their early life (Brown et al., 2015). As a result of the risk-adverse phenotype, studies have reported significant increases in survival in both laboratory and field experiments. This increase in survival was attributed to the behavioural aspects that risk-adverse individuals display (i.e., less bold and decreased swimming activity when exposed to novel stimuli) (Ferrari et al., 2015a; Ferrari et al., 2015b). However, being risk-averse is only beneficial for short periods of time, as prolonged aversion to risk will lead to loss of fitness promoting behaviours (i.e., foraging).

In addition to behavioural changes, prey have a myriad of strategies in terms of shape and colouration to combat the threat of predators. These range from body armour (O'Brien et al., 1979), mimic colouration (Eagle and Jones, 2004), visual interferences through the use of lines (Barlow, 1972), and false eyespots, also known as ocelli (Brakefield et al., 1996; Stevens, 2005). The ocellus is used as a diversion technique to draw attacks of the predator towards non-vital regions of the body (Neudecker, 1989). As predators initiate an attack, they anticipate the movement of prey. Therefore, the ocellus may cause the predator to misjudge the direction of the prey's escape path, resulting in an unsuccessful capture (Powell, 1982). A successful attack by a predator on the posterior end (i.e., where the ocellus is located) of the prey may allow the target to escape and survive (McPhail, 1977; Powell, 1982) as non-vital organs are damaged in the process. A previous study by Lönnstedt et al. (2013) on juvenile ambon damselfish, *Pomacentrus amboinensis*, found individuals who were exposed to predator cues (i.e., visual, olfactory or a combination of the two) grew deeper bodies or had larger ocellus sizes. Fish exposed to predator cues also exhibited a higher survival in the field with 10 % compared to 60% in individuals with no predator experience. This points towards plasticity within the morphological make up of juvenile coral reef fish. These morphological changes occurred as a response to the risk they were exposed to in their environment after settlement.

This study aims to investigate how risk history and experience with predators affect the morphometrics of a developing juvenile coral reef fish during the first four weeks after settlement. Specifically, the study aimed to determine whether the history of risk early in settled life affected morphological development, and whether this effect was modified by subsequent exposure to predator risk later in settled life. As exposure to high-risk conditions affect the phenotype, a difference may be seen in the morphometrics (i.e., body size, eye diameter and ocellus diameter and area) of individuals raised in high-risk conditions compared to low-risk counterparts. Additionally, these changes may vary during the early stages post-settlement as individuals grow in their varying risk regimes.

3.3 Methods

Study Species

The study species was the ambon damselfish, *P. amboinensis* (Pomacentridae), a reef-associated planktivore commonly found on reefs throughout the Indo-Pacific. Juveniles were caught overnight using light traps placed approximately 100m apart and 30-50m from the reef edge (Meekan et al., 2001), off Lizard Island Research Station (LIRS) (14°40’S, 145°28’E), Great Barrier Reef, Australia in November 2014. Fish caught from the light trap were then transported back to the laboratory where 48 *P. amboinensis* were placed in groups of 6 in a series of eight 3 L flow-through plastic tanks. All fish were fed to satiation with newly hatched *Artemia spp*. three times per day.

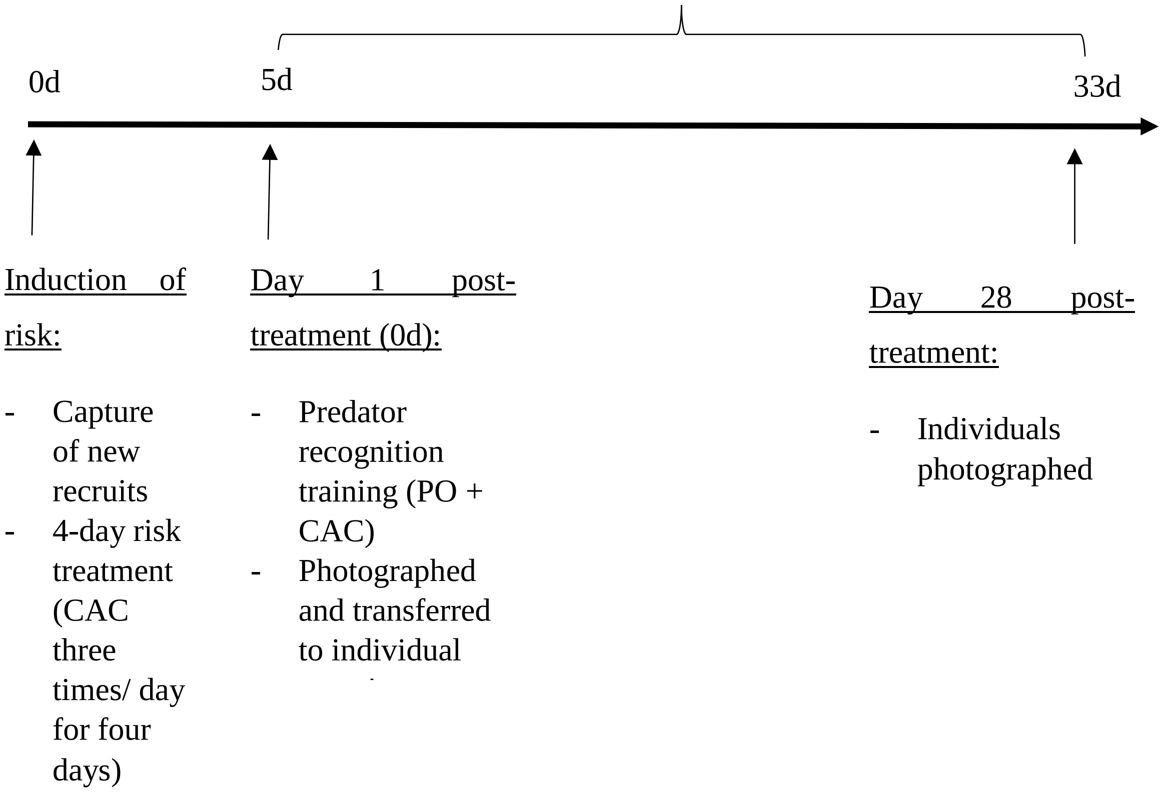
The predator used in this study was the brown dottyback, *Pseudochromis fuscus*. Predators were caught using a weak solution of clove oil and hand nets whilst SCUBA diving on the reefs around LIRS (Munday and Wilson, 1997). After capture, *P. fuscus* were transported back to the lab and kept in 32 L flow through tanks where they were fed to satiation with squid twice daily.

Experimental Design

Background risk levels were manipulated following a well-established procedure (Chivers et al., 2014; Ferrari et al., 2015a). Briefly, fish were exposed to one of two treatments: a low-risk treatment (n=24) or a high-risk treatment (n=24) for four days (Fig. 3.1). To obtain a high-risk background, fish were exposed to chemical alarm cues at three random times throughout the day. The injections of chemical alarm cue took place between 0800 and 1800h, with a minimum of 1.5h between injections to allow the cue to dissipate and flow out of the tank. The alarm cue solution was prepared just prior to being used, by making vertical cuts on each side of 2 freshly-euthanized donor conspecific fish and then rinsing the fish in 60 mL of seawater. Subsequently, 10 mL of the alarm cue solution was injected into the conditioning tanks, giving us a concentration of 2 cuts/L once injected. This concentration has been shown to elicit strong anti-predator responses in coral reef fish (Chivers et al., 2014; Ferrari et al., 2015a). Low-risk conditions were obtained by following the same procedure as above but injecting 10 mL of seawater rather than alarm cue.

Following 4 days of high-/low-risk conditioning, half of each risk-history treatment were then trained to either identity the odour of *P. fuscus* as risky, while the other half were trained to identify the odour as being non-risky. To allow fish to recognise the predator as risky, groups of 6 fish were placed into 32 L tanks, with an airstone without flow through seawater. Subsequently, 30 mL of predator odour (PO) and 15 mL of chemical alarm cue (obtained from 4 euthanized fish) were injected into the tank. PO was prepared by turning off water flow in the predator’s tank for 6 h to allow the predator’s odour to accumulate in the water before 30 mL of water was taken out. Chemical alarm cues were prepared using the same method from the high-risk conditioning phase. To allow fish to recognise the predator as safe, saltwater (SW) was injected instead of chemical alarm cue, as prey fish will not label an odour as a risk in the absence of chemical alarm cues. After 30 mins, the flow of water was turned on and individual *P. amboinensis* were transferred to a plastic bag partly filled with seawater, photographed (Canon G1x) against a 1x1 cm grid, and then transferred to a 5 L plastic container of flowing seawater where they were housed for the next 28 days. Each day, individual fish were fed 5 mL of *Artemia nauplii* (~200 *Artemia* /mL) three times per day and had 5 mL of PO injected into their container three times a day. The injections of PO took place between 0800 and 1800h, with a minimum of 1.5h between injections to allow the cue to dissipate and flow out of the tank. A 1h period between feeding and PO injections was used to avoid overlapping information. Fish were photographed on days 0 and 28. In this way, Risk history (High or Low) was crossed with Predator history (PO or SW) in a 2 x 2 design (n = 10). Characteristics were measured from the photographs using Image-J (www.nih.com): body depth; standard length, diameter of eye, diameter of ocellus and area of ocellus.

(A) High-risk + learned predator:



Induction of risk:

- Capture of new recruits
- 4-day risk treatment (CAC three times/ day for four days)

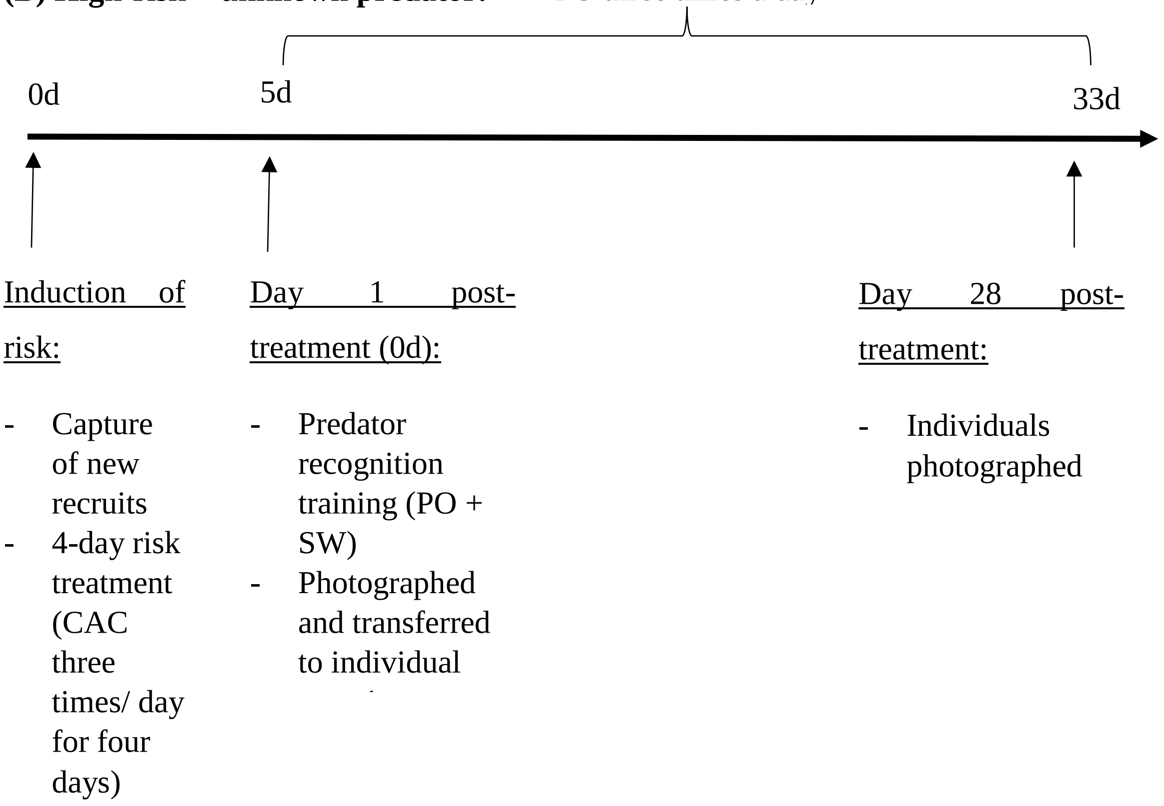
Day 1 post-treatment (0d):

- Predator recognition training (PO + CAC)
- Photographed and transferred to individual

Day 28 post-treatment:

- Individuals photographed

(B) High-risk + unknown predator:



Induction of risk:

- Capture of new recruits
- 4-day risk treatment (CAC three times/ day for four days)

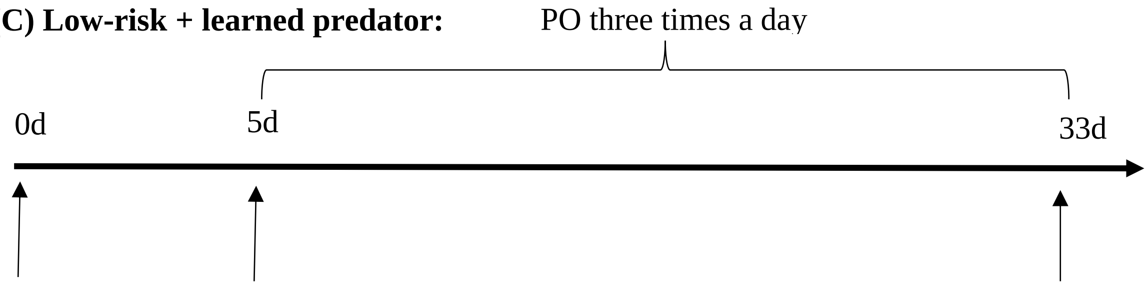
Day 1 post-treatment (0d):

- Predator recognition training (PO + SW)
- Photographed and transferred to individual

Day 28 post-treatment:

- Individuals photographed

(C) Low-risk + learned predator:



Induction of risk:

- Capture of new recruits
- 4-day risk treatment (SW three times/ day for four days)

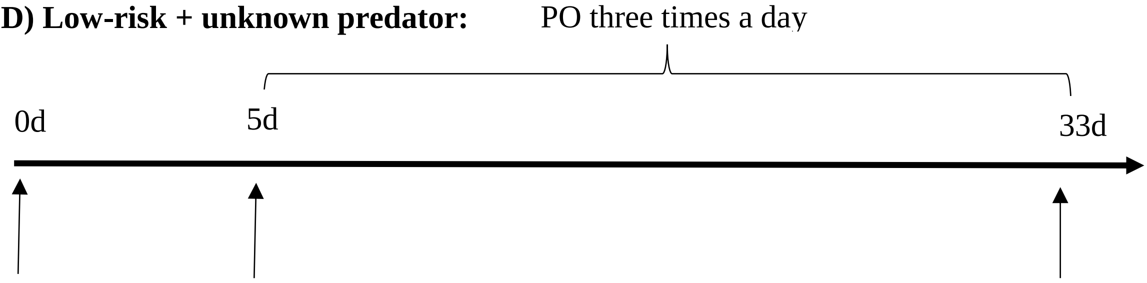
Day 1 post-treatment:

- Predator recognition training (PO + CAC)
- Photographed and transferred to individual

Day 28 post-treatment:

- Individuals photographed

(D) Low-risk + unknown predator:



Induction of risk:

- Capture of new recruits
- 4-day risk treatment (SW three times/ day for four days)

Day 1 post-treatment:

- Predator recognition training (PO + SW)
- Photographed and transferred to individual containers

Day 28 post-treatment:

- Individuals photographed

Figure 3.1: Experimental timeline to assess how varying risk conditions and predator experience affect morphometrics in *P. amboinensis*.

Statistical Analysis

All data was analysed using the statistical software STATISTICA 7.0 and checked for normality and homogeneity of variance before statistical analysis.

A two-factor analysis of covariance (ANCOVA) using standard length as a covariate was undertaken to determine if there was a difference between the fixed factors Risk history and Predator history after risk history treatment, 0d, and 28d. Body depth did not significantly contribute to the model ($p > 0.05$).

3.4 Results

Univariate analyses showed that risk history had an effect at 0d and 28d but not predator experience. Specifically we see risk history affected ocellus size at 0d (Table 3.1) and the eye and ocellus sizes at 28d, but did not affect body depth (Table 3.2). At 0d, after risk history treatment, individuals from high-risk history treatments already possessed a larger area and diameter of ocellus (Fig. 3.2). Diameter of the eye was smaller under high-risk, while diameter of the ocellus, and area of the ocellus were larger under high-risk (Fig. 3.3A,B,C). Consequently, the proportion of the eye to ocellus diameter was also affected by risk, with fish with a low-risk history possessing an eye diameter that represented 57.9 % of the diameter of the ocellus, while only representing 47.7% of the ocellus size under high-risk (Fig. 3.3D).

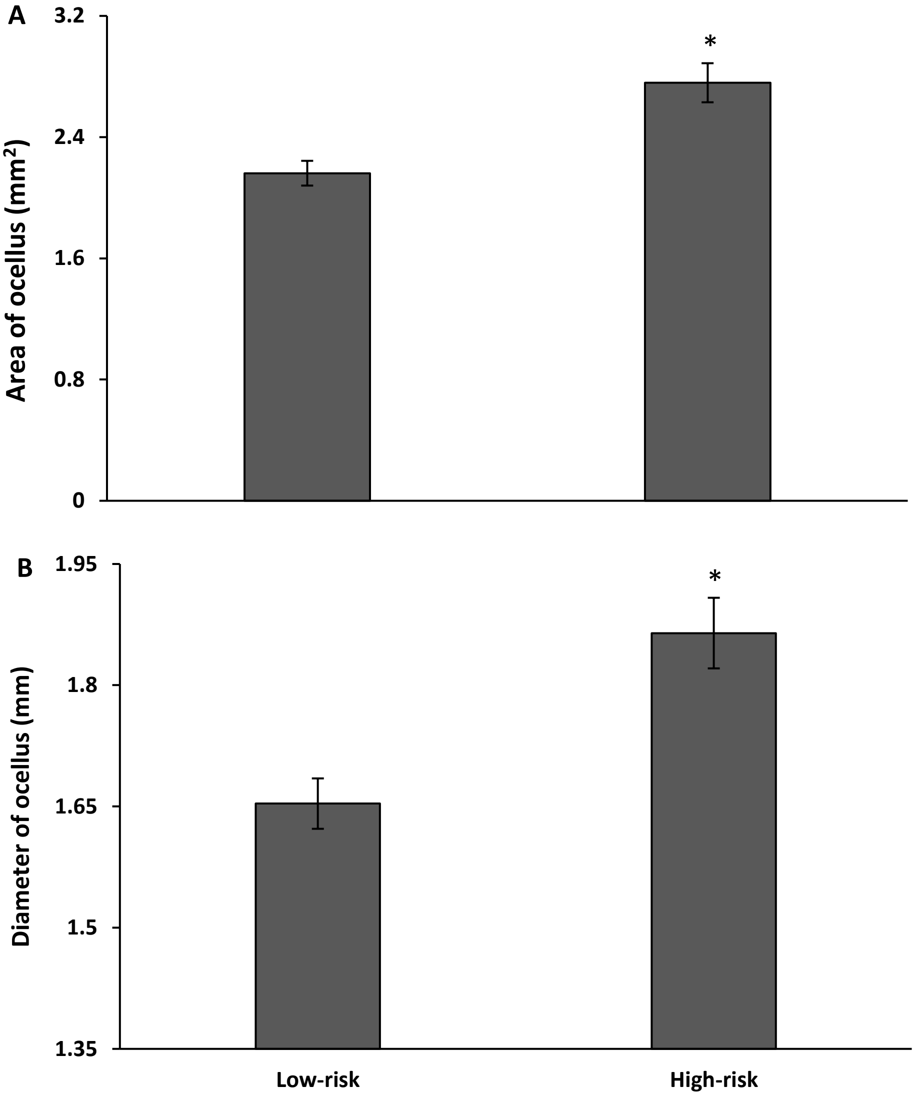


Figure 3.2: Morphology of juvenile *P. amboinensis* at 0 days post risk history treatment (± SE). Area of ocellus (mm^2) (A); diameter of ocellus (mm) (B).

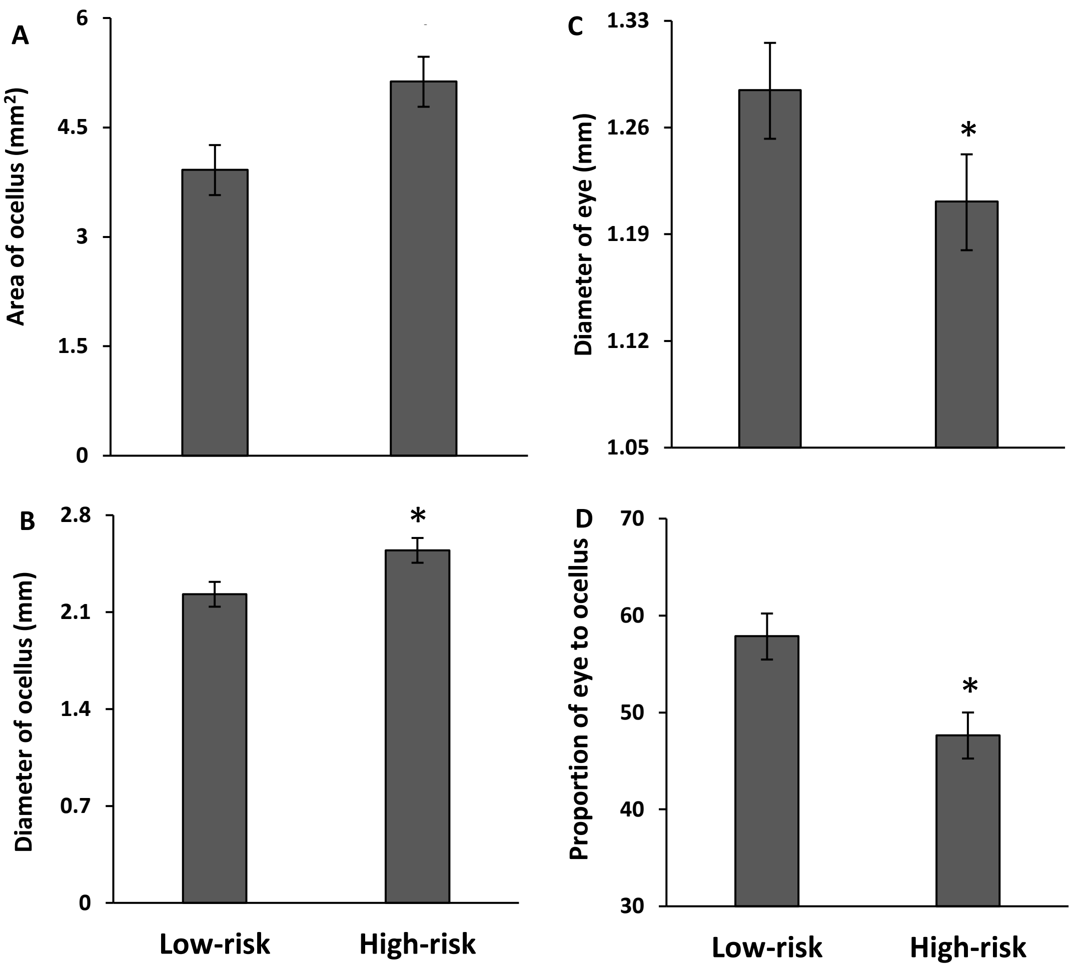


Figure 3.3: Morphology of juvenile *P. amboinensis* at 28 days post risk history treatment (± SE). Area of ocellus (mm^2) (A); diameter of ocellus (mm) (B); diameter of eye (mm) (C); proportion of the diameter of the eye to the ocellus (D).

Table 3.1: Two-way ANOVA values for morphometric variables of juvenile *P. amboinensis* exposed high-/low-risk conditions and taught to either recognise or not recognise a predator at 0 days post risk history treatment. Degrees of freedom are given in brackets.

	Risk history (1, 36)		Predator history (1, 36)		Risk x Predator (1, 36)	
Variable	F	p	F	P	F	P
Ocellus diameter	5.44	**0.025**	0.21	0.65	2.24	0.14
Area of ocellus	5.42	**0.026**	0.16	0.69	0.06	0.80

Table 3.2: Two-way ANOVA values for morphometric variables of juvenile *P. amboinensis* exposed high-/low-risk conditions and taught to either recognise or not recognise a predator at 28 days post risk history treatment. Degrees of freedom are given in brackets.

	Risk history (33)		Predator history (33)		Risk x Predator (33)	
Variable	F	p	F	P	F	P
Eye diameter	9.12	**0.005**	1.56	0.22	0.14	0.71
Ocellus diameter	19.93	**<0.0001**	0.22	0.64	0.07	0.79
Area of ocellus	19.80	**<0.0001**	0.39	0.54	2.57	0.12
Eye/Ocellus diameter	31.22	**<0.0001**	41.84	0.15	3.66	0.65
Body depth/ Standard length	3.35	0.08	7.90	0.18	2.1	0.49

3.5 Discussion

High risk early in life has been shown to cause the development of a risk-averse phenotype (Brown et al., 2014; Chivers et al., 2014; Ferrari et al. 2015a; Ferrari et al., 2015b). When stresses occur in early life they can have a lasting effect that is amplified with development and growth. Metamorphosis may be one such period of extreme sensitivity to the conditions faced by organisms with complex life cycles. The life cycles of these individuals are composed of a number of disparate life stages. The present study suggests that the period immediately after settlement is particularly important for establishing developmental trajectories of morphology. Fish exposed to high-risk in the first four days after settlement had larger ocelli than those exposed to low-risk conditions. After 28 days, high-risk fish had smaller eyes and larger ocelli compared to those raised in low-risk conditions. Interestingly however, fish exposed to low risk for the first four days, followed by high-risk from a known predator subsequently did not develop a high risk morphology. This reinforce Ferrari et al.'s (2015a) finding that they could only induce partial neophobia in damselfish 10 days after settlement. Our research stresses the importance of events during the days immediately following settlement to ecological facets that may have important ramifications for their interaction with predators.

The window of risk sensitivity appears to be coincident with metamorphosis in our damselfish. Little is known about the details of the remodelling that occurs coincidently with settlement from the larval to benthic juvenile life phase (Leis and McCormick, 2002, McMenamin and Parichy, 2013). For all fish, it represents a rapid shift in morphology and colouration to varying degrees (McCormick et al., 2002). For many it represents a major change in the alimentary tract (e.g., Randall, 1961), orientation and morphology of the jaw (e.g., Labelle and Nursall, 1985; McCormick, 1999), and major changes to the sensory systems (McCormick and Shand, 1993). Damselfishes undergo a major shift in metabolism, going from being a larvae with a very high metabolism to a hypoxia tolerant juvenile (Nilsson et al., 2007). Given the dramatic shift in many aspects of physiology immediately after settlement, it is easy to see why current risk during that transitional phase could canalise growth and physiological trajectories which continue to have influences later in life.

The current study showed that juvenile damselfish raised under high-risk history exhibited smaller eyes and larger ocelli compared to counterparts raised in low-risk history by the end of the experiment. This points towards a strategy utilised by prey to confuse predators during phases of the predator-prey interaction, namely detection and evasion (Lima and Dill, 1990). The large ocellus size while coupled with a small eye, follows the “deflective hypothesis” where the ocellus is used as a diversion technique used to draw attacks of the predator towards non-vital regions of the body (Neudecker, 1989). Other hypotheses that explain the purpose of a larger eyespot compared to the true eye are the “intimidation hypothesis” and the “reaction distance hypothesis” (Meadows, 1993). The “intimidation hypothesis” suggests that predators may be intimidated by perceiving that the prey is larger than it is and thus reduce the likelihood of an attack. This hypothesis came from studies that showed insects with eyespots that are much larger than their true eye and mimic the eye structure of vertebrates (Neudecker, 1989; Meadows, 1993). Hence, the eyespot would intimidate potential predators. The “reaction distance hypothesis” suggests that the presence of a large eyespot may deceive predators into initiating an attack from a further distance than normal (Meadows, 1993). This would allow prey sufficient time to initiate a response. This is seen in a previous study by Blest (1957) where butterflies with eyespots elicited four times more successful escape responses compared to butterflies lacking eyespots. The growth of the larger ocellus and smaller eye would likely give high-risk fish an advantage in predator-prey interactions compared to low-risk counterparts.

These results support a study conducted by Lönnstedt et al. (2013) whereby juvenile *P. amboinensis* exhibited smaller eyes and larger ocelli when housed with *P. fuscus* for a six week period. The decrease in eye diameter in response to predators has also been documented for a number of species. For example, Zaret and Kerfoot (1975) found a species of water flea, *Bosmina longirostris*, living in areas with high levels of predation displayed a significantly smaller eye-pigmentation diameter compared with conspecifics living in areas with no predators. Conversely, a study carried out on 97 species of birds by Moller and Erritzoe (2010) found the eye size in birds to increase with elevated predator pressure. A large eye would be beneficial in open habitats but in coral reef systems where visual cues can be limited, due to topographical complexity, other senses are needed to gain information (i.e. olfaction) (McCormick and Lönnstedt, 2013). These are among many studies investigating the effects of risk on morphology using predator cues (Clark and Havell, 1992; Chivers et al., 2008). This study shows that individuals are able to display similar plasticity in their morphometrics through their perceived risk levels in the absence of predator cues. This is displayed through risk conditioning, through the use of alarm cues, being a major factor in results.

A recent study by Brown et al. (2015) using juvenile convict cichlids, *Amatitlania nigrofasciata*, found neophobic behaviour towards novel stimuli is lost with repeated exposures through a process known as neophobic extinction. False threats are relabelled as safe whereas real threats are affirmed through processes such as associative and social learning. In this experiment, prey were only exposed to a learned or unknown predator odour three times a day without the addition of alarm cues for the duration of the experiment. Similar to Brown and colleague’s study, the threat of the predator may have been downgraded over time with repeated exposures, although the threat is likely to have been maintained for at least two of the four weeks (Lienart, 2016). This may explain why measures such as body depth are not significantly different between risk conditions as seen in Lönnstedt et al. (2013). Despite this, individuals raised within high-risk conditions exhibited differences in their ocellus size four days post-settlement. This suggests that fish are placed on a growth trajectory dependent on experiences gained from the period immediately post-settlement. This is demonstrated through risk conditioning rather than learned predator experience, presenting the greatest effect on the morphometrics of juvenile damselfish used in this experiment.

Following on from this study, future studies should investigate the advantage of prey exhibiting smaller eyes in the face of increased risk regimes. This could be attributed to two strategies; firstly, to draw more attention toward the ocellus (Neudecker, 1989) or the reallocation of resources toward an improved olfactory system to aid in predator detection. This could be answered with histological analysis of the gustatory and olfactory systems of prey fish. As the sense of sight may be reduced due to a smaller eye, prey may invest more energy towards other senses such as taste and smell to aid them in identifying potential predators. A possible factor in the results seen in this study (i.e. the non-significant difference in body depth) may be the affect neophobic extinction played on prey. Therefore, studies assessing how frequency and timing of cue exposures accelerate the process of neophobic extinction may be useful to gain a timeframe of the neophobic phenotype in juvenile coral reef fish. Finally, further investigation on ontogenetic differences of growth trajectories in response to risk regimes would be useful to ascertain whether varying risk conditions in the period post-settlement affect resulting morphology in adult fish.

Chapter 4: General Discussion

Organisms in most environments are frequently at risk from predators throughout their lives. Prey are most vulnerable during the early stages of life for both terrestrial (insects, Rosenheim et al., 1993; mammals, O'Donoghue and Boutin, 1995) and marine animals (invertebrates, Gosselin and Qian, 1997; fish, Almany and Webster, 2006). On coral reefs, risk is particularly high during settlement when young fish are unfamiliar to the dangers around them (Lönnstedt et al., 2012). While processes that affect the survival of eggs, embryos and larvae are critically important as the initial inputs to cohort success, understanding the processes that affect survival over the settlement transition for fishes will better enable us to understand the balance of factors that regulate populations and communities on the coral reef. By investigating the development and implications of a risk-averse phenotype, this thesis explores an important mechanism whereby fishes may influence their probability of survival in a high risk environment.

High-risk environments and their effect

The complex life histories of most fishes on coral reefs result in early larval life stages that have little opportunity to gain knowledge of the predators they will interact with once they have metamorphosed and reached the next stage in life. Recent research suggests that there is potentially some level of parental transfer of information (Atherton and McCormick, submitted), and this may affect settlement choices at small spatial scales (Vail and McCormick, 2011), but the importance of these information sources is as yet unknown. Because small predators are numerous on coral reefs (Holmes and McCormick, 2010) and patchily distributed (Shulman, 1985), the best and most reliable information source will be obtained by the individual. It is this strong selection pressure to avoid the wrong choice under the threat of predation that has resulted in a sophisticated system of rapidly learning, categorising and prioritising threats (reviewed by Chivers and Smith, 1998; Ferrari et al., 2010). One reliable indicator of risks are chemical alarm cues, which are released through damage, and sensitivity to these species-specific chemicals appears to develop during embryogenesis, immediately after the development of olfactory capabilities (Atherton and McCormick, 2015). These cues are likely used to gauge the risk of particular habitat patches at the time of settlement, which is typically during the night (Dufour et al., 1996; Leis and McCormick, 2002).

As risk is variable over spatial and temporal scales, animals need to adapt to the level of risk posed by the habitats they live in. On coral reefs, fishes settling onto reefs with high topographic complexity may be exposed to less risk than those settling on reefs with less shelter (Shulman, 1985; McCormick & Lönnstedt, 2013). Additionally, a juvenile fish settling onto a reef at the beginning of the season (i.e., residents) may be exposed to less risk than those settling later in the season as they outcompete and push newly settled “intruders” away from shelter (Poulos and McCormick, 2015; Geange et al., 2016). Therefore, individuals need to match their antipredator response to their perceived levels of risk. Fishes in low-risk habitats get the opportunity to learn their potential predators over time. For those that settle into high-risk habitats, the importance of learning quickly can be the difference between survival and death (McCormick and Holmes, 2006; Lönnstedt et al., 2012). Thus, the development of a risk-averse phenotype in these high-risk habitats allows an individual the time to survive their initial encounters with novel predators (Ferrari et al., 2015a; Ferrari et al., 2015b).

The plasticity shown in fish raised in high-risk habitats may increase the probabilities of survival through becoming risk-averse (Chivers et al., 2014; Ferrari et al., 2015a; Ferrari et al., 2015b; Ferrari et al., 2015c; Chapter 2; Chapter 3). Laboratory experiments have shown that post-settlement, predator-naïve juvenile fishes exposed to a short duration of high-risk conditions exhibit similar (Chivers et al., 2014; Ferrari et al., 2015a; Chapter 2 [turning angle]), or greater, antipredator responses (Chapter 2 [response distance]; Chapter 3) to risk cues as experienced counterparts. The altered morphology and enhanced escape responses demonstrated in this thesis may in part explain the higher survival documented in fishes raised in high-risk conditions in laboratory and field studies (Lönnstedt et al., 2013; Ferrari et al., 2015a; Ferrari et al., 2015b). These changes exhibited by individuals with the risk-averse phenotype allow them to influence interactions with their predators.

Predator-prey interactions follow a series of steps in which prey have to use mechanisms or behaviours to avoid or escape their predator. Chapter 3 showed that fishes exposed to high-risk conditions grew larger ocelli and smaller eyes compared to those in low-risk. This may be a strategy to combat predators both before and after the initiation of a strike. The role of false eye spots has been studied in both marine and terrestrial environments (Neudecker, 1989; Stevens, 2005). It is believed that prey use these false eye-spots to intimidate predators through their resemblance to the eyes of larger organisms (Chaetodontids, Neudecker, 1989; Lepidopterans, Stevens, 2005). Predators are much less likely to attack a forewarned prey because of the higher likelihood that the prey will initiate an effective escape (Smith and Smith, 1989), so while the prey may actually be unaware of the presence of a motivated predator, the false-eye spots may trick the predator into not engaging in its final strike. Any reduction in the number of escape responses a prey has to initiate will be energetically beneficial because escape responses are anaerobically fuelled and energetically expensive (Moyes et al., 1993). In the event of an unsuccessful escape response, the secondary role of the ocellus is to confuse the predator and divert attacks towards non-vital organs (Neudeceker, 1989; Stevens, 2005), or a direct the strike in a way that facilitates prey escape (Kjernsmo and Merilaita, 2013). Research has shown that the development of larger eyespot to eye proportion results in higher survival in terrestrial (Stevens, 2005), freshwater (Kjernsmo and Merilaita, 2013) and marine environments (Lönnstedt et al., 2013).

A recurrent finding in these studies is the reduction in the absolute size of the eye relative to the size of the prey fish (Kjernsmo and Merilata, 2013; Lönnstedt et al., 2013; Chapter 3). This is interesting because there is a positive relationship between lens diameter and visual acuity (Shand, 1997), suggesting that with the reduction in eye diameter comes a reduction in visual acuity. This is surprising given that the visual system, like most sensory systems such as the balance and hearing system (Campana, 1984), or gustatory system (McCormick, 1993), are conserved under environmental stress (Pankhurst, 1992) due to their importance in normal life processes. If fish are reducing their visual acuity as a result of predation risk, the finding suggests that obtaining a larger ocellus relative to the eye has a consistent and strong selection advantage.

One variable that was not significantly different between high- and low-risk fish was body depth (Chapter 3). This was interesting given that Lönnstedt et al. (2013) found *Pomacentrus amboinensis* juveniles to grow deeper bodies when exposed to predator cues. This has also been found in freshwater systems. Crucian carp, *Carrassius carassius*, developed deeper bodies in the presence of predator cues from northern pike, *Esox lucius*, and alarm cues from conspecifics (Brönmark and Petterson, 1994). Additionally, grey treefrogs, *Hyla versicolor*, grew deep tail fins and shorter bodies when exposed to larval predators (Relyea and Hoverman, 2003). The lack of response in body depth, in Chapter 3, may have been due to the threat of predatory cues being downgraded over the experimental period (Lienart, 2016). Brown et al (2015a; 2015b) also found neophobic behaviour towards novel stimuli is lost with repeated exposures through a process known as neophobic extinction.

In Chapter 2, short term elevated risk resulted in individuals enhancing their escape responses. As prey need to match their antipredator responses to their perceived level of risk, we saw a trend whereby individuals displayed higher escape speeds, turning angles, response distances and a shortened latency. Although this was a short term experiment, the plasticity of risk-averse fishes may see the growth of deeper bodies with increased muscle mass over longer time periods. This increase in muscle mass would result in higher speeds, acceleration and turning rates which would increase the probability of escaping predatory attacks (Domenici et al., 2008).

Ontogeny and frequency of exposure to cues affect the risk-averse phenotype

This thesis found that the period immediately following settlement was particularly sensitive to risk. The perceived levels of risk during this period are then used to develop behaviour and growth trajectories. Due to variable temporal risk, individuals may need to develop a risk-averse phenotype a few days after settlement. However, Ferrari et al. (2015a) found 10 day post-settlement whitetail damselfish, *Pomacentrus chrysurus* exhibited weaker risk-averse responses compared to fish exposed to elevated risk immediately after settlement. As individuals get older, the need for risk-averse responses may decrease as vital information, such as predator identity, is learnt through time and experience. Therefore, the benefits of being risk-averse decrease and the associated costs increase. Brown and colleagues (2015a; 2015b) showed the extinction of risk-averse behaviours after repeated exposures to a harmless cue. For example, the behaviour of juvenile convict cichlids, *Amatitlania nigrofasciata*, returned to control levels (i.e., high feeding rates and activity) after 11 days of exposure to a initially novel cue (Brown et al., 2015a). Similarly, Trinidadian guppies, *Poecilia reticulata*, showed a decrease in risk-averse behaviour towards a novel odour after five repeated exposures (Brown et al., 2015b).

Responses of fish to high-risk treatments in Chapters 2 and 3 may have been weakened due the frequency (Chapter 2) and ontogeny (Chapter 3). In Chapter 2, the fishes used (spiny chromis. *Acanthochromis polyacanthus)* were ~45 post-hatching. Given the older age, the effect of high-risk conditions may have been weakened as a trade-off to acquire enhanced escape response may not be needed as individuals may be large enough to escape most predators. Additionally, *A. polyacanthus* juveniles have very different life histories compared to the complex life histories of other fishes and amphibians tested. As juveniles maintain close proximity to their parents post-hatching and lack a planktonic larval stage, information on risks within that habitat may be acquired through social learning from their parents. Thus, the development of a risk-averse phenotype may not be under strong selection. In Chapter 3, fishes were exposed to predator cues three times a day for the duration of the experiment. The risk associated with the predator odour may have decreased due to the lack of a corresponding risk cue (i.e. chemical alarm cue). Lienart (2016) found that response of the neon damselfish, *Pomacentrus coelestis* towards a learned predator waned after 14 days of not being reinforced as a threat. It is likely this reduction in antipredator responses may have been attributed to individuals labelling cues as safe through latent inhibition (Mitchell et al., 2011). Therefore, we may have seen stronger results if a new novel cue was used.

The developmental window

The results in Chapter 3 point towards a window of time directly following a settlement transitional period that is vital to the subsequent success of individuals. During this period, individuals determine the relevant behaviours and morphology needed to survive in their habitat. This may either lead to a life where learning can be acquired through time (i.e., low-risk habitats), or a period of time with a risk-averse phenotype, which will safeguard individuals from death (i.e., high-risk habitats). In Chapter 3, we saw that four days of high-risk exposure was enough to set individuals on a growth trajectory that resulted in larger ocelli and smaller eyes, along with slightly larger bodies. Fishes used in Chapter 2 may have missed the window, and thus, displayed less of a reaction towards threat cues.

The growth trajectory determined by perceived risk levels during the developmental window was not altered by subsequent risk cues (i.e., learned predator odour). This inflexibility is interesting as there must be a cost associated with it (i.e., decreased foraging). For example, woodfrog tadpoles, *Lithobates sylvaticus*, exposed to predators grew larger but developed less rapidly (Relyea, 2002). As the risk-averse behavioural phenotype is temporary (Brown et al., 2015a), fish may place as much resources available towards growth early on in order to eliminate the threat of gape-limited predators that prey upon juvenile fish such as, the brown dottyback, *Pseudochromis fuscus* (Holmes and McCormick, 2010). Therefore, costs or compensatory growth may follow after the risk-averse phenotype becomes extinct and fish are at relatively lower risk than they were as newly settled juveniles. As risk-averse fish eat less because they lower feeding rates when exposed to novel cues (Brown et al, 2014; Chivers et al., 2014; Ferrari et al., 2015a), they may have the energy resources available to put towards growth and other processes. Therefore, they may undergo a period of compensatory growth after they do not require to be risk-averse and favourable conditions are restored, though such compensatory growth tends to come at a cost later in life (Ali et al., 2003).

This developmental window is an important period for prey individuals with complex life-histories. As risk is variable spatially and temporally, prey are not able to predict the level of risk they will be exposed at each ontogenetic stage. Therefore, the ability to modulate their phenotype should occur at key life-history transitions. The associated costs of inflexibility to spatial and temporal risk in terms of growth trajectories may only be temporary as compensatory growth can make up for the initial costs.

Future directions

The results from this thesis help us to further understand the effects of high-risk conditions; however, they only shed light upon individual effects. Future studies should focus upon varying risk conditions (i.e., 2, 8 and 12 days of high-risk) on morphological and physiological traits of fishes. Investigating how body morphology that has been affected by risk alters escape responses will help link information gained from Chapters 2 and 3. As the risk-averse phenotype is not permanent, studies should look to understand the timeframe in which behavioural responses return to control levels and their capacity to compensate for potentially maladaptive phenotypes under changing risk conditions. Specifically, is it age or frequency of exposure to cues that degrades the risk-averse response? Finally, to determine the cost of this early inflexibility (Chapter 3), studies should look at the effects of being risk-averse after the extinction of the behavioural response.

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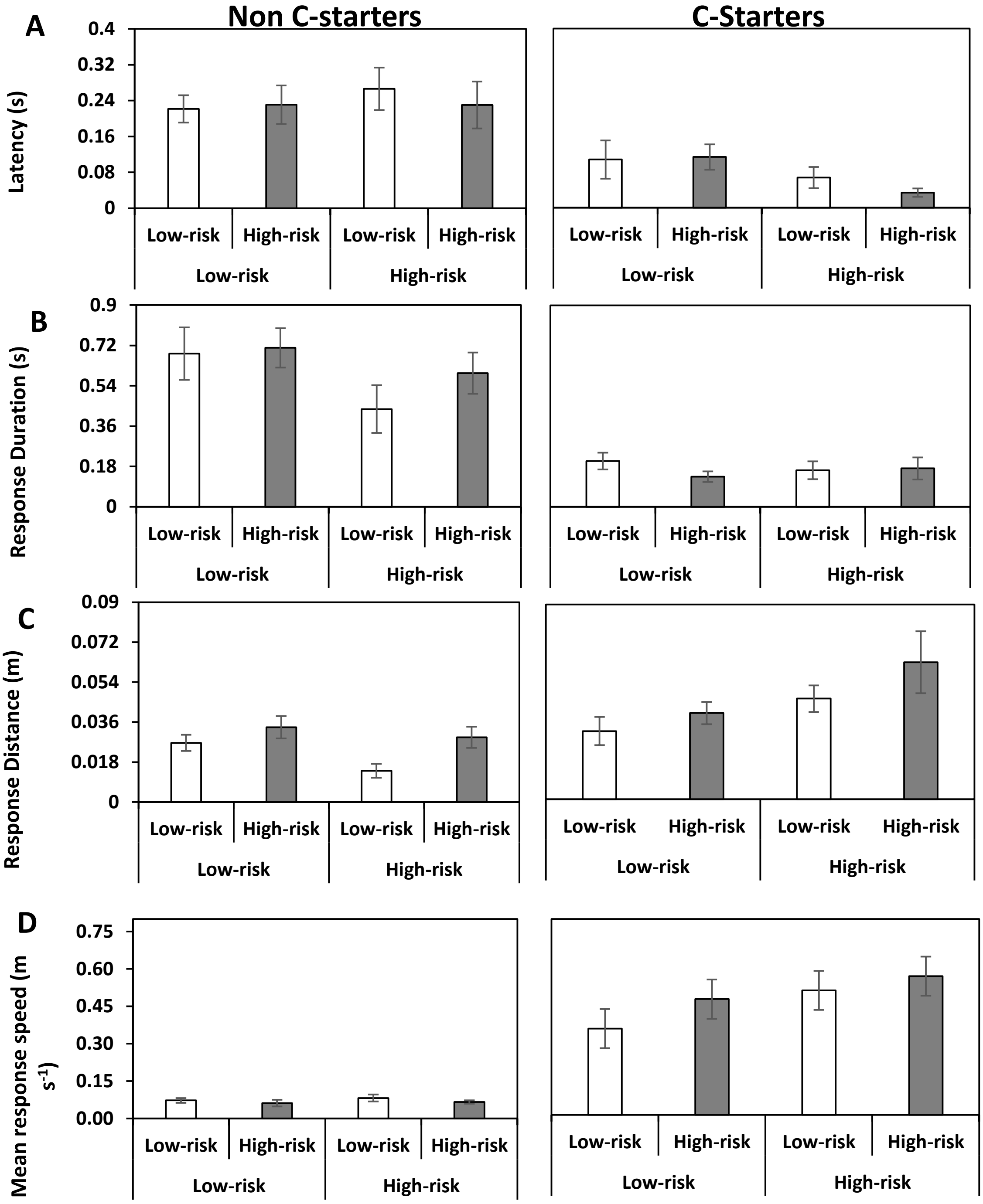
Appendix



Supplementary figure 1: Mean ±SE latency to respond (A), response duration (B), escape distance (C) and mean escape speed (D). Fish were maintained under low-risk or high-risk conditions for 4 days (background risk), and were then exposed to an acute stressor (low-risk – empty bars, or high-risk – grey bars) minutes prior to being startled.

Supplementary figure 2: *A. polyacanthus* juvenile used in Chapter 2. Dianne J. Bray. *Acanthochromis polyacanthus* in Fishes of Australia. Retrieved 30 Jan 2017, http://fishesofaustralia.net.au/home/species/311.

Supplementary figure 3: *P. amboinensis* juvenile used in Chapter 3. Dianne J. Bray. *Pomacentrus amboinensis* in Fishes of Australia. Retrieved 30 Jan 2017 from http://fishesofaustralia.net.au/home/species/2336.